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Design, Synthesis and Application of New Heterobifunctional Photoaffinity Probe for the Studies of Protein-Protein Interactions involved in the Actin-linked Calcium-regulated System of Muscle Contraction

by

© Pele Choi-sing Chong

A THESIS

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External Examiner

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海天遼闊立多時

唐君毅

DEDICATION

Follow your dream...
    take one step at a time
and don't settle for less,
    just continue to climb.
Follow your dream...
    if you stumble, don't stop
and lose sight of your goal,
    press on to the top.
For only on top
    can we see the whole view,
    can we see what we've done
    and what we can do,
    can we then have vision
    to seek something new...
Press on,
    and follow your dream.
    Amanda Bradley

To my teachers, brothers, sisters, mother and Ming Kit.

ABSTRACT

In the rabbit skeletal muscle system previous studies suggested that sulfhydryl (SH) groups might be in the proximity of the sites of interaction of the following protein complexes: troponin C - troponin I, troponin-tropomyosin, actin-actin, actin-tropomyosin, and actin-myosin. Therefore a bifunctional cross-linking reagent that could be specifically attached to these SH groups would be invaluable for the identification of the proteins in the vicinity of the labelled SH group and enable the determination at the molecular level of amino acid residues involved in the site of interactions. This information would aid us in understanding how the Ca^{2+}-induced conformational changes in troponin C can be transmitted from troponin C to all other components of the regulatory complex. For this reason we have designed and synthesized a new heterobifunctional photoaffinity probe, N-(4-azidobenzoylglycyl)-S-(2-thiopyridyl)cysteine (AGTC) from cysteine via a coupling of the N-hydroxysuccinimide ester of 4-azidobenzoylglycine to S-(2-thiopyridyl)cysteine. The chemical stability and reactivity of AGTC have been characterized. AGTC is readily dissolved in an aqueous buffer at pH 7.5 and is stable at room temperature ranging in pH from 3 to 9. The disulfide bridge moiety of AGTC is stable to the conditions of photolysis used to activate the arylazido group for crosslinking. AGTC is readily incorporated (90%-100%) within 2 hr into such proteins as

rabbit skeletal troponin C, tropomyosin, and actin through disulfide bridge formation. The degree of incorporation of AGTC into proteins can be monitored by spectrophotometric determination of the release of pyridine-2-thionine at 343 nm. AGTC has a cross-linking distance of 14 Å. The aryl azide moiety is inert until photolysis, permitting the removal of the excess reagents from the modified proteins and control experiments to ensure the correct protein-protein interaction. Also the aryl azide is nonspecific, in that it does not require the presence of a particular reactive functional group at the binding site for cross-linking to occur. Demonstration of the general utility of AGTC to study protein-protein interactions has been carried out in three different protein complexes: the interactions involving troponin C and troponin I, tropomyosin and troponin, and the subunits of troponin complex.

Troponin C was labelled specifically at cysteine 98 with radioactive AGTC to form AGC-TnC which was used to form a binary complex with S-carboxamidomethylated troponin I (CM-TnI) in benign media. Photolysis of CM-TnI-AGC-TnC complex resulted in the formation of a 1:1 covalently cross-linked complex in 30% yield. The radiolabelled CM-TnI-AGC was isolated from the cross-linked complex by reduction of the disulfide bridge between AGC and TnC using DEAE-Sephadex chromatography in the presence of 8M urea and 1 mM EGTA. These results indicated that CM-TnI was within 14

Å of cysteine 98 of TnC.

AGC-TM (AGTC was attached to cysteine 190 of TM via disulfide bond formation) was used to determine which component of rabbit skeletal troponin (CM-Tn) was in close proximity to cysteine 190 of TM. Photolysis of the CM-Tn-AGC-TM complex in the presence of Ca^{2+} resulted in formation of a 1:1 covalently cross-linked complex in 7% yield. The radiolabelled troponin (CM-Tn-AGC) was isolated by hydroxylapatite chromatography. CM-Tn-AGC was further separated into its individual components on DEAE-Sephadex chromatography. Radioactive measurements and SDS-urea gel electrophoresis indicated that only troponin T (TnT) was radiolabelled. A limited (15 min) chymotryptic digest of CM-Tn-AGC resulted in the isolation of T2-AGC (residues 159-259 of TnT). More extended proteolysis of CM-Tn-AGC allowed the isolation of T2'-AGC (residues 159-227 of TnT). When the CM-Tn-AGC-TM complex was photolyzed in the absence of Ca^{2+} compared to the presence of Ca^{2+} there was 1.7 fold increase in the cross-linking yield. This result suggested that there was a Ca^{2+}-sensitive conformational change in the binding region of TnT around cysteine 190 . A tightening of the complex about cysteine 190 in the absence of Ca^{2+} could explain the decrease in reaction of the arylnitrene with solvent. Nevertheless, region 159-227 of TnT is in the vicinity of cysteine 190 of TM in both the presence and absence of Ca^{2+}.

The topographical relationship of the SH groups of rabbit skeletal troponin has been investigated to provide information for the specific labelling of the SH groups with AGTC for the purpose of monitoring the Ca^{2+}-induced conformational changes in the troponin complex. The approach involved the reaction of ^{14}C-iodoacetamide with SH groups in native troponin and various binary complexes of troponin components in the presence and absence of Ca^{2+}. The SH groups involved in interaction sites and those exposed were identified by peptide mapping using two dimensional paper electrophoresis and autoradiography. In the presence and absence of Ca^{2+} cysteine 133 of TnI in native troponin was exposed while cysteines 48 and 64 of TnI and cysteine 98 of TnC were inaccessible to modification. Differential labelling of the TnI-TnT complex showed that cysteine 133 of TnI was again exposed and cysteines 48 and 64 were inaccessible. ^{14}C-S-carboxamidomethylation of the TnI-TnC complex showed that all three cysteine residues of TnI (48, 64, and 133) were accessible to modification in the absence of Ca^{2+} while cysteine 48 and 133 were only partially accessible in the presence of Ca^{2+}. Cysteine 98 of TnC was inaccessible to modification in both the presence and absence of Ca^{2+}. These studies indicated that in native troponin, TnT was solely responsible for the inaccessibility of cysteines 48 and 64 of TnI in absence of Ca^{2+}, and 64 of TnI in the presence of Ca^{2+}. The inaccessibility of cysteine 48 of TnI in native troponin in the presence of Ca^{2+} may be

due to a combined effect of conformational changes in TnI induced by TnC upon Ca^{2+} binding and the interaction with TnT. These studies have provided the information for selective and specific attachment of AGTC into TnI for the study of Ca^{2+}-induced conformational changes in the troponin complex itself or troponin in the thin filament.

AGTC was attached to cysteines 48 and 64 of skeletal TnI to determine which component of troponin was in close proximity to these cysteines. The reconstituted troponin complex (AGTC labelled CM-TnI, TnT, and TnC) was photolyzed and separated using DEAE-Sephadex chromatography in the absence of reducing agent. Radioactive measurements indicated that 12% of the cross-linker reacted with solvent and 88% with proteins. The percentage radiolabel found in TnI, TnI-TnT, and TnI-TnC complexes was 35%, 55%, and 10%, respectively. These results have indicated that both TnT and TnC are in the vicinity of one or both cysteines 48 and 64 of TnI. Of the total radiolabel found in TnT, 33% and 23% was located in two CNBr fragments, CB4 (residues 176-230) and CB2 (residues 71-151). The most likely interpretation of the cross-linking results is that one of the interaction sites between TnI and TnT is an ionic interaction involving the region around cysteines 48 and 64 of TnI (residues 28-82) with the CB5 region of TnT (residues 135-185).

Finally, combining the present photochemical cross-linking results and all other studies, a working model of the regulatory complex (TM-Tn) was constructed to aid us in future experimental design to expand our knowledge on how the Ca^{2+}-induced conformational changes in TnC can be transmitted from TnC to all other components of the regulatory complex.

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ABBREVIATIONS

A	Absorbance at the given wavelength
AC	N-(4-azido-[1-^{14}C]-benzoyl)cysteine
Actomyosin ATPase	The actin activated Mg^{2+}-dependent hydrolysis of ATP by myosin
ADP	Adenosine-5'-diphosphate
ADP.Pi	Adenosine-5'-diphosphate with non-covalently bound phosphate
AGC	N-(4-azidobenzoyl-[2-^{3}H]-glycyl)cysteine
AGC-(CM-TnI)	AGC attached to rabbit skeletal S-carboxamidomethylated TnI at cysteines 48 and 64 through disulfide bond formation
AGC-TM	AGC attached to α-tropomyosin at cysteine 190 through disulfide bond formation
AGC-TnC	AGC attached to rabbit skeletal TnC at cysteine 98 through disulfide bond formation
^{14}C-AGC	N-(4-azido-[1-^{14}C]-benzoylglycyl) cysteine
AGTC	N-(4-azidobenzoyl-[2-^{3}H]-glycyl)-S-(2-thio pyridyl)cysteine
^{14}C-AGTC	N-(4-azido-[1-^{14}C]-benzoylglycyl)-S-(2-thiopyridyl)cysteine
AMP	Adenosine-5'-monophosphate
ATC	N-(4-azido-[1-^{14}C]-benzoyl)-S-(2-thio pyridyl)cysteine
ATP	Adenosine-5'-triphosphate
β-MeOH	β-mercaptoethanol
CB 1	Cyanogen bromide fragment (residues 1-151) of TnT
CB 2	Cyanogen bromide fragment (residues 71-151) of TnT
CB 4	Cyanogen bromide fragment (residues 176-230) of TnT

CB 9	Cyanogen bromide fragment (residues 84-135) of TnC
CM	S-carboxamidomethyl
CM-Tn	S-carboxamidomethylated rabbit skeletal troponin
CM-Tn-AGC	S-carboxamidomethylated rabbit skeletal troponin with AGC covalently attached by photochemical cross-linking
CM-Tn-AGC-TM	The covalently cross-linked complex of CM-Tn and TM resulting from photolysis
CM-TnI	S-carboxamidomethylated rabbit skeletal troponin I
CNBr	Cyanogen bromide
COOH-terminal	Carboxyl terminal of polypeptide chain
Cys	Cysteine
DCC	Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
D_2O	Deuterium water
d^3H_2O	Doubly deionized and distilled water
DFP	Diisopropyl fluorophosphate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediamino-tetra-acetic disodium salt
EGTA	Ethylene glycol-bis-(β-aminoethylether) -N,N,N',N'-tetraacetic acid
F-actin	Fibrous polymer of G-actin
g	Grams
G-actin	Globular monomers of actin
HMM S-1	Heavy meromyosin subfragment-1
Hse	Homoserine
IR	Infrared

M.W.	Molecular weight
Mg^{2+}-ATPase	The ATPase of myosin which is activated by actin
NH-terminal	Amino terminal of polypeptide chain
nm	Nanometers
NMR	Nuclear magnetic resonance
NPTM	Non-polymerizable tropomyosin
PMSF	Phenymethylsulfonyl fluoride
S-1	Soluble single headed fragment of myosin
SDS	Sodium dodecyl sulfate
T2	α-chymotryptic fragment (residues 159-258) of TnT
T2'	α-chymotryptic fragment (residues 159-227) of TnT
T2-AGC	T2 of TnT with AGC covalently attached by photochemical cross-linking
TM	α-tropomyosin
TM-AGC	Tropomyosin with AGC covalently attached by photochemical cross-linking
Tn	Rabbit skeletal troponin
TnC	Calcium binding subunit of troponin
TnC-AGC-(CM-TnI)	The convalently cross-linked complex of TnC and AGC-(CM-TnI) resulting from photolysis
TnI	Inhibitory subunit of troponin
TnI-AGC	Troponin I with AGC covalently attached by photochemical cross-linking
TnT	Tropomyosin binding subunit of troponin
TnT-AGC	Troponin T with AGC covalently attached by photochemical cross-linking
TnT-AGC-(CM-TnI)	The covalently cross-linked complex of TnT and AGC-(CM-TnI) resulting from photolysis

TPCK	Tosylphenylalanine chloromethyl ketone
Tris	Tris-(hydroxymethyl)aminomethane
UV	Ultraviolet
η	The relative viscosity

I. INTRODUCTION

Everyday, the muscles in our bodies are repeatedly contracting and relaxing as we carry out our daily activities. The moment we appreciate the ability of muscle is when we see the flight of birds, run a 100 meters dash, or hear the beat of rock music. All of these are possible because most muscles have very low standby power, that can be switched within 1 msec to controlled levels of force and cause movement at controlled speeds. How do muscles, molecular machines, convert chemical-bond energy (ATP) into coordinated motion? The molecular mechanism by which the transduction process (conversion of chemical energy to mechanical energy) occurs is one of the most interesting and challenging questions in molecular biology today. This transduction mechanism occurs not only in muscle, but also in such events as cell division, and the beating of cilia and flagella of bacteria. Therefore, the study of muscle contraction is a study of life!

To identify the contractile mechanism has been the subject of research for over four decades. Using various biophysical and biochemical techniques, some basic aspects of the structure of muscle are now available. To aid in our understanding of muscle contraction, the following excellent reviews can be referred to: Adelestein and Eisenberg(1980); McCubbin and Kay(1980); Perry(1979); and Weber and Murry(1973). Also, a more detailed description of the molecular basis of muscle contraction can be found in

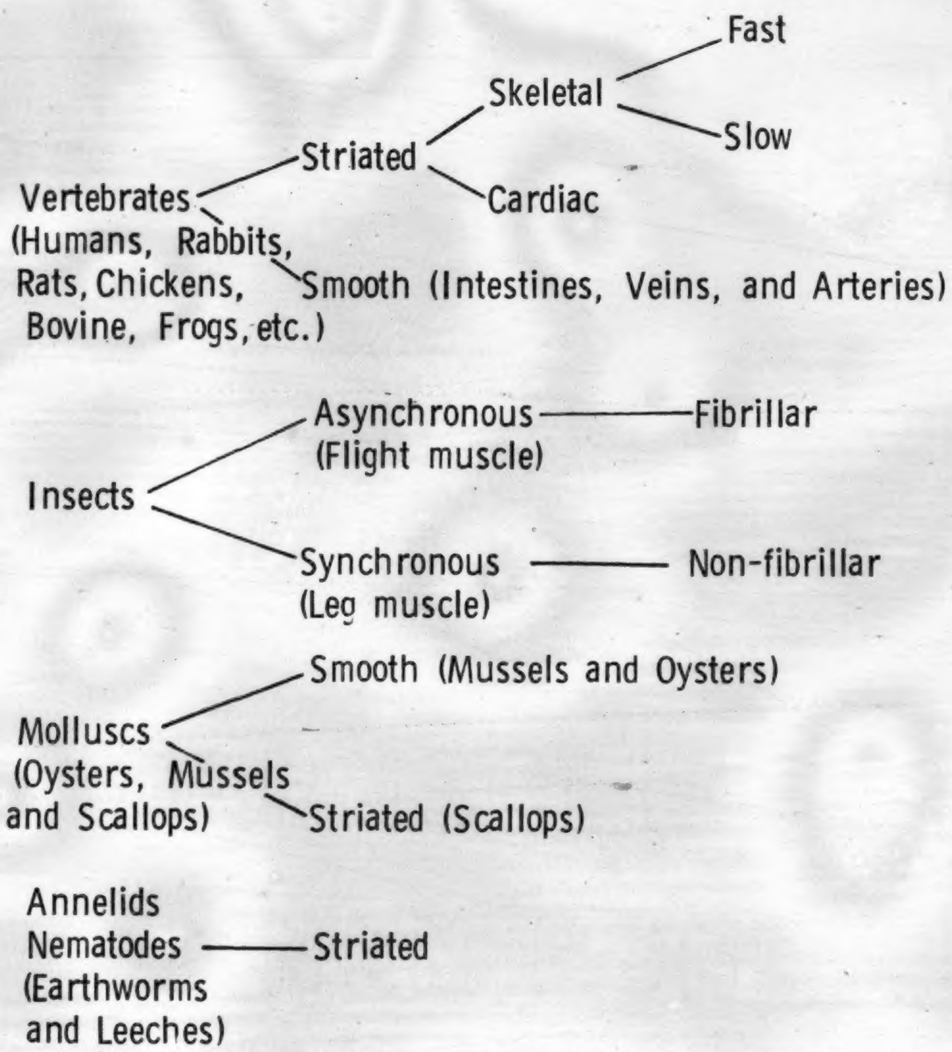

Fast
Skeletal
Slow
Striated
Cardiac
Vertebrates
(Humans, Rabbits,
Rats, Chickens,
Bovine, Frogs, etc.)
Smooth (Intestines, Veins, and Arteries)
Asynchronous
(Flight muscle)
Fibrillar
Insects
Synchronous
(Leg muscle)
Non-fibrillar
Smooth (Mussels and Oysters)
Molluscs
(Oysters, Mussels
and Scallops)
Striated (Scallops)
Annelids
Nematodes
(Earthworms
and Leeches)
Striated

Fig. 1.1 Classification of the major muscle types used in muscle research (Adapted from Squire, 1981).

Squire(1981), Lehnigner(1975) and Stryer(1981).

A. Ultrastructure of Muscle

It is common knowledge that if a muscle is stimulated electrically it will shorten or contract. Although most muscles demonstrate this phenomenon, by no means are they all identical in structure. In fact, the muscles from different sources or in different parts of the same animal are specialized for particular functions ranging from the rapid movement required in the wings of insects to the prolonged closure of the shells of clams. The common and specialized features of different muscles have been reviewed by Squire(1981). The classification of the major muscle types used in current research is summarized in Fig 1.1. Since rabbit skeletal muscle was used throughout in my research projects, the following sections will concentrate on the structure of vertebrate skeletal muscle together with an account of some of the current ideas on the mechanism of force generation.

1. Fiber Types

Different types of fibers in vertebrate skeletal muscle are distinguished by the kind of innervation that they receive. In some fibers, the fiber membrane is excitable as is the nerve membrane, and the action potential generated at the neuromuscular junction is propagated along the length of the fiber so that the whole of it contracts in an all or

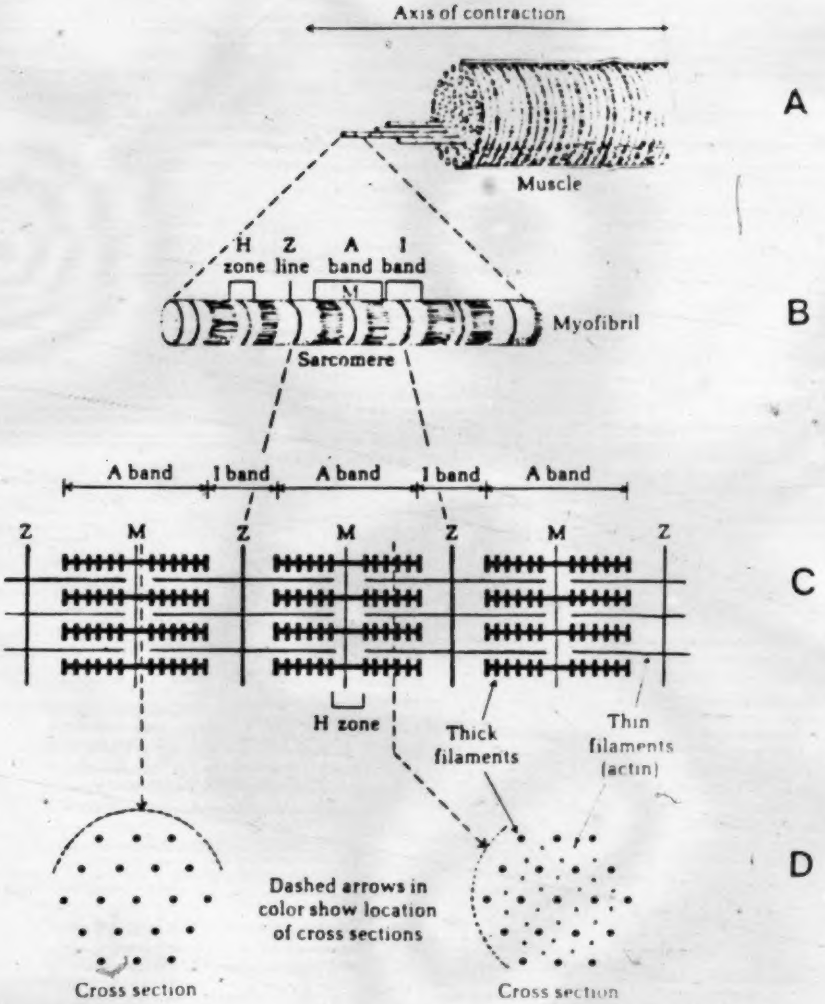

Axis of contraction
A
Muscle
H zone
Z line
A band
I band
M
B
Myofibril
Sarcomere
A band
I band
A band
I band
A band
Z
M
Z
M
Z
M
Z
C
H zone
Thick filaments
Thin filaments (actin)
D
Dashed arrows in color show location of cross sections
Cross section
Cross section

Fig. 1.2. Gross structure of striated muscle. (A) Cross section of a muscle fiber. (B) Enlargement of one myofibril showing the repeating sarcomeres. (C) Schematic illustration representing the thick and thin filaments of sarcomeres. (D) Cross section of sarcomere. [From Lehninger, 1975]

none fashion. The twitch fibers in the skeletal muscle (such as the sartorius) are one example. Other fibers (such as tonic fibers) have membranes that are electrically inexcitable and the action potential at the neuromuscular junction only causes a localized stimulation of the contractile apparatus. Therefore a single stimulus has relatively little effect, but repetitive stimulation can cause the gradual build up of tension. On the basis of their response to stimulation, twitch and tonic fibers are sometimes referred to as fast and slow fibers respectively.

2. Myofibrils

Now let us take a look at the organization of the contractile apparatus. A contractile unit is a long and multinucleated cell. Bundles of these individual muscle cells compose the muscle fiber. The fibers are surrounded by plasma membranes called sarcolemmas which are in close association with nerves (Fig. 1.2A). Dissection of a muscle fiber (or cell) reveals the basic contractile elements, the myofibrils, which are arranged in parallel bundles in the axis of contraction (Fig. 1.2B). Each myofibril contains many myofilaments (Fig. 1.2C) and is surrounded by a specialized membrane system called the sarcoplasmic recticulum. Also, myofibrils are bathed by the intracellular fluid called sarcoplasm. The sarcoplasm contains energy sources such as glycogen, glycolytic enzymes, ATP, ADP, AMP, phosphate, phospho-creatine, creatine and metal ions

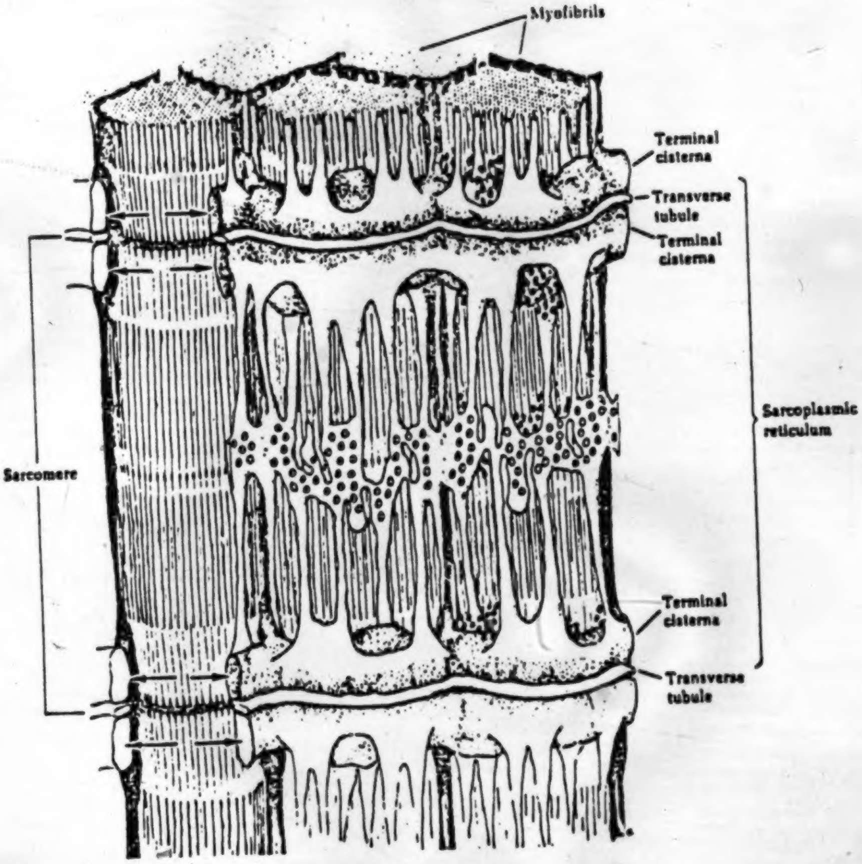

Myofibrils
Terminal cisterna
Transverse tubule
Terminal cisterna
Sarcoplasmic reticulum
Sarcomere
Terminal cisterna
Transverse tubule

Fig. 1.3. T-system and Sarcoplasmic reticulum. The electron micrograph is of frog sartorius muscle. The drawing shows the vertical bundles which are individual myofibrils and the positions of transverse tubules and sarcoplasmic reticulum. [Adapted from Peachey, 1965]

(potassium, calcium, magnesium). Mitochondria and myoglobin (the oxygen transporter) are also present in the sarcoplasm. A communication system has been set up to link the innervation between sarcolemma (receiving nerve impulse) and the sarcoplasmic recticulum (the place where Ca^{2+} ions are released and trigger muscle contraction) via transverse tubules called T-tubes (Fig. 1.3).

3. Sarcomere

Closer inspection of the myofibril interior under a phase contrast microscope reveals the characteristic alternating light and dark bands which account for the name "striated" muscle (Fig. 1.2C). The bands arise due to repeating units of two distinct types of filaments called thick and thin filaments. The light bands are called isotropic or I-bands, because their physical properties are uniform regardless of the direction the measurement is taken. I-bands are about 1000 nm long and attached to a dense line 50 nm thick called the Z-line. The dark bands are called anisotropic or A-bands, because they show double refraction indicating asymmetric molecules which are oriented in one specific direction. When the muscle fibers are at rest the A-bands are about 1600 nm long and are bisected by a dense line called M-line (Fig. 1.2C). One repeating unit, from Z-line to Z-line, is called a sarcomere about 2500 nm long.

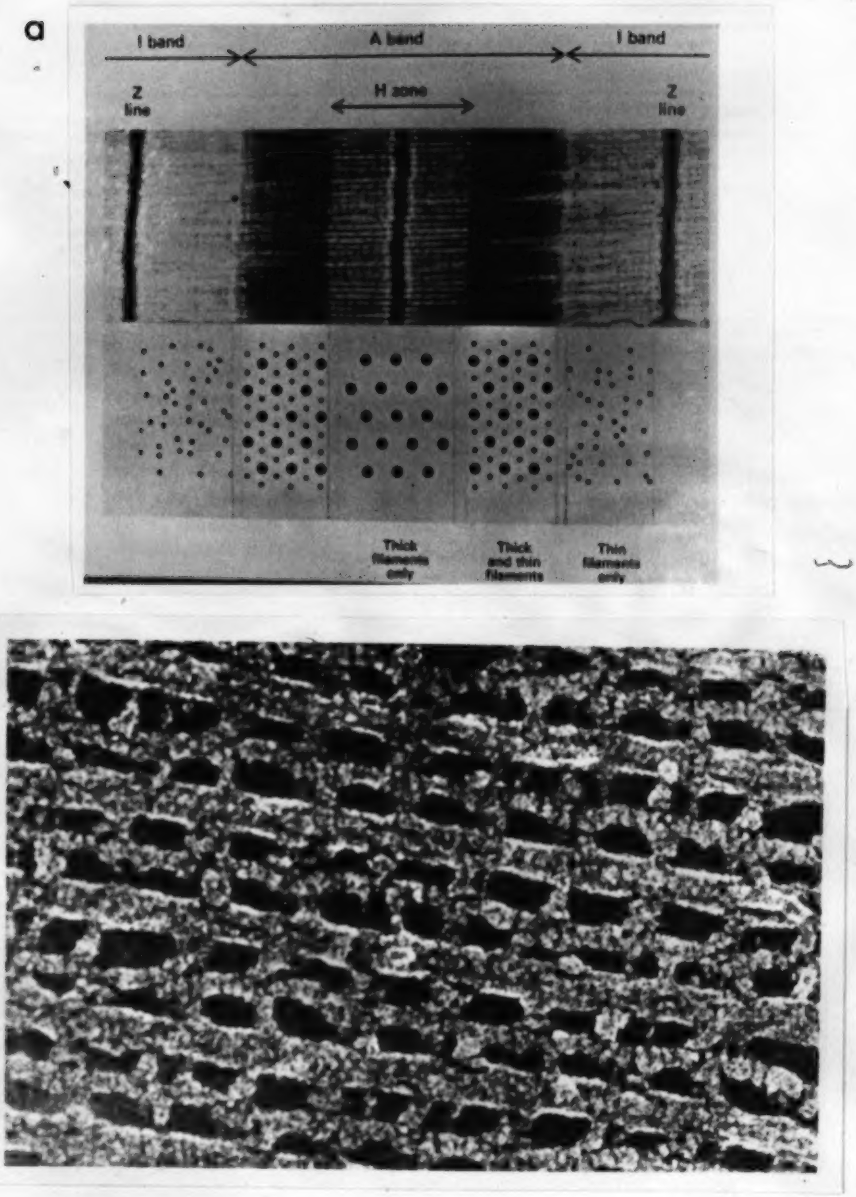

a
I band
A band
I band
Z line
H zone
Z line
Thick filaments only
Thick and thin filaments
Thin filaments only
b

Fig. 1.4. (a) Electron micrograph of a longitudinal section of a skeletal muscle myofibril. Schematic diagrams of cross sections are shown below the micrograph. [Courtesy of Dr. Hugh Huxley]

(b) Electron micrograph showing cross-bridges between thick and thin filaments. [Courtesy of Dr. John Heuser]

Furthermore, A-bands have been shown to be composed of both thick and thin filaments, 16 nm and 6 nm in diameter respectively (Fig. 1.4a). In the cross section of A-bands, it can be seen that each thick filament is surrounded by six hexagonally arrayed thin filaments whereas each thin filament is surrounded by three triangularily arranged thick filaments. The electron micrograph (Fig. 1.4b) has revealed short projections between the thick filaments to the thin filaments. These connections are called crossbridges. Thus, the thick filaments are present throughout the entire A-band whereas the thin filaments begin at the Z-line and overlap partially into the A-band (Fig. 1.2C and Fig. 1.4a).

4. Sliding Filament Model

From careful microscopic measurements of the length of the sarcomere reveal that sarcomere shortens by as much as a third of its original length as muscle contracts. However, the A-bands always remain constant in length while the I-bands can disappear in a strong contraction. This led to the "Sliding-Filament Model" (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954) which states:

i) The lengths of the thick and thin filaments do not change during muscle contraction.

ii) Instead, the length of the sarcomere decreases during contraction because the two types of filaments overlap more. Thick and thin filaments slide past each other in contraction.

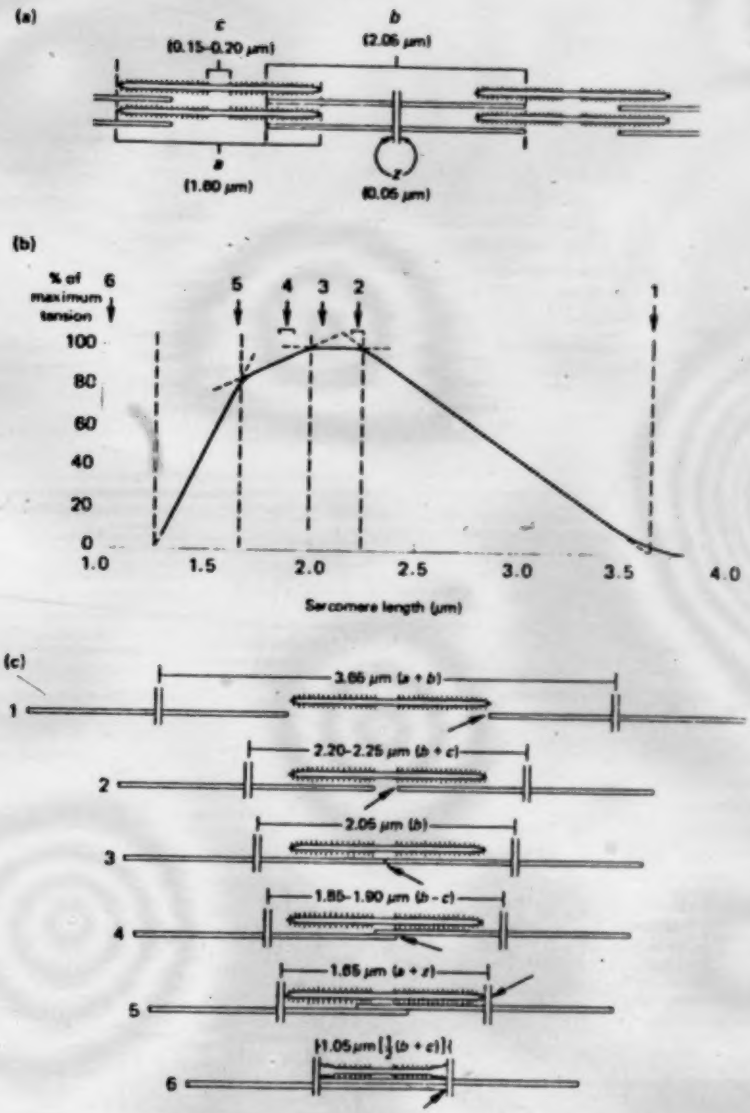

(a)
c
(0.15–0.20 μm)
b
(2.06 μm)
a
(1.60 μm)
z
(0.05 μm)
(b)
% of maximum tension
6
5
4
3
2
1
100
80
60
40
20
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Sarcomere length (μm)
(c)
1
3.65 μm (a + b)
2
2.20–2.25 μm (b + c)
3
2.05 μm (b)
4
1.85–1.90 μm (b – c)
5
1.65 μm (a + z)
6
1.05 μm [½(b + c)]

Fig. 1.5. Sarcomere length and tension development.

(a) Dimensions of thick and thin filaments and regions.

(b) Tension delevlopment versus distance between adjacent Z-lines. Numbers above curve refer to (c).

(c) Diagrams showing degree of overlap. [From Gordon et al., 1966]

iii) The force of contraction is generated by a process that actively moves one type of filament past the neighboring filaments of the other type. This suggests that the crossbridges between the thick and the thin filaments in the dense portions of the A-bands are rapidly formed and broken as the filaments slide along each other (Fig. 1.5).

TABLE 1-I

The Protein Composition in Rabbit Skeletal Muscle[a]

Location	Proteins	% of total protein in the myofibrils	Molecular weight
Thick Filaments	Myosin[b]	54	450,000
	C-protein	3.5	140,000
	M-line protein I		155,000
	M-line protein II		88,000
Thin Filaments	Actin[c]	20-25	42,000
	Tropomyosin[d]	7	66,000
	Troponin[e]	7	70,000
Z-line	α-Actinin		100,000
	β-Actinin		70,000

[a] Myosin, actin, tropomyosin and troponin are the major and important proteins involved in muscle contraction.

[b] Myosin consists of two heavy chains and four light chains.

[c] Under physiological conditions actin molecules are polymerized to form F-actin.

[d] Tropomyosin consists of two α-helical chains in coiled-coil fashion.

[e] Troponin consists of three subunits: troponin C (M.W. 18,000), troponin I (M.W. 21,000), and troponin T (M.W. 30,500).

B. Molecular Structure of Muscle Proteins

Before a clear understanding of the mechanism of muscle contraction can be reached, the individual proteins making up these filaments must be discussed. Fig. 1.6 illustrates a close look at the arrangement in the thick and thin filaments. The composition and physical properties of the major muscle proteins are summarized in Table 1-I.

1. Thick Filaments

Hanson and Huxley (1957) have shown that the thick filament is composed of a protein called myosin. Myosin is highly asymmetrical and made up six polypeptide chains: two heavy chains and four light chains (Fig. 1.6b). Myosin is an enzyme which is able to hydrolyze ATP (ATPase). Each of the heavy chains has molecular weight 200,000. They coil about one another at their carboxyl terminal regions to form a two stranded α-helical coiled-coil . The amino terminals are globular in shape and have the ability to hydrolyze ATP in the presence of the light chains.

Not all four light chains are essential for the ATPase activity. The nonessential light chains are known as DTNB light chains, because these light chains (M.W. 18,000) can be removed from the globular head of heavy chains by treatment with 5,5'-dithiobis-(2-nitrobenzoic acid). On the other hand, the two essential light chains are known as A-1 (M.W. 21,000) and A-2 (M.W. 16,500), because they can only be removed from the heavy chains by treating with alkali

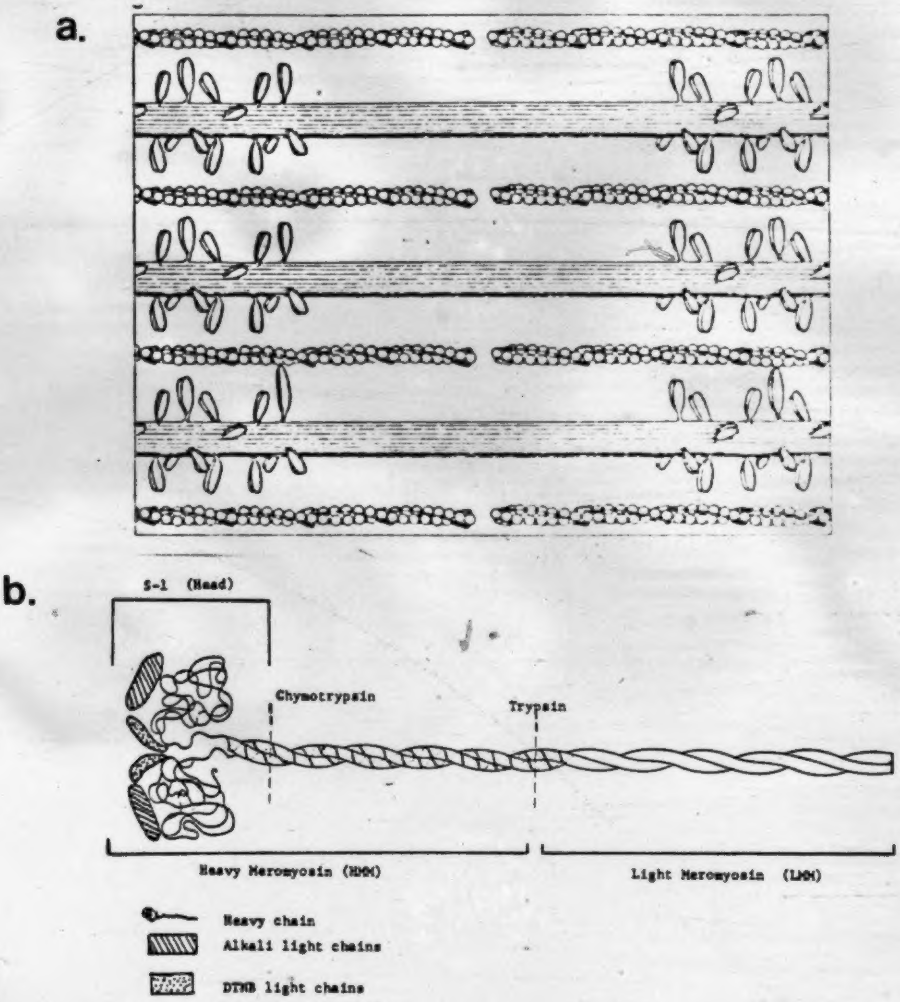

a.
b.
S-1 (Head)
Chymotrypsin
Trypsin
Heavy Meromyosin (HMM)
Light Meromyosin (LMM)
Heavy chain
Alkali light chains
DTNB light chains

Fig. 1.6. (a) Model for the arrangement of the myosin molecules in the thick filament illustrating their biploar arrangement. [From Cohen, 1975]

(b) Schematic diagram of myosin molecule. The molecule is composed of six polypeptide chains: two heavy chains and two pairs of light chains. S-1, HMM, LMM are fragments generated by limited proteolysis at the points indicated. [From Cote, 1980] solution.

In high salt (0.6M KCl) myosin is monomeric; however at the physiological ionic strength (0.15M KCl) myosin aggregates to form bipolar thick filaments spontaneously. The rod portion of the heavy chains form the core of the filament. The globular heads protrude from the core and are hexagonal around it (Fig. 1.6a).

Myosin can be specifically cleaved at several sites (as indicated in Fig. 1.6b) to yield single-headed S-1 or double-headed heavy meromyosin (HMM) fragments. Both of these fragments are soluble at low salt concentration (50 mM KCl) and also retain the ability to hydrolyze ATP (Lowey et al., 1969).

Recent research has revealed that the thick filaments also contain two other proteins, namely C-protein and M-line protein, Their functions are not well understood, but it has been suggested that these two proteins serve as a clamp to hold the bundle of myosin molecules together at the M-lines (Fig. 1.2).

2. Thin Filaments

The thin filament is composed of three principle proteins: actin, tropomyosin, and troponin. A schematic molecular structure of the thin filament is shown in Fig. 1.7.

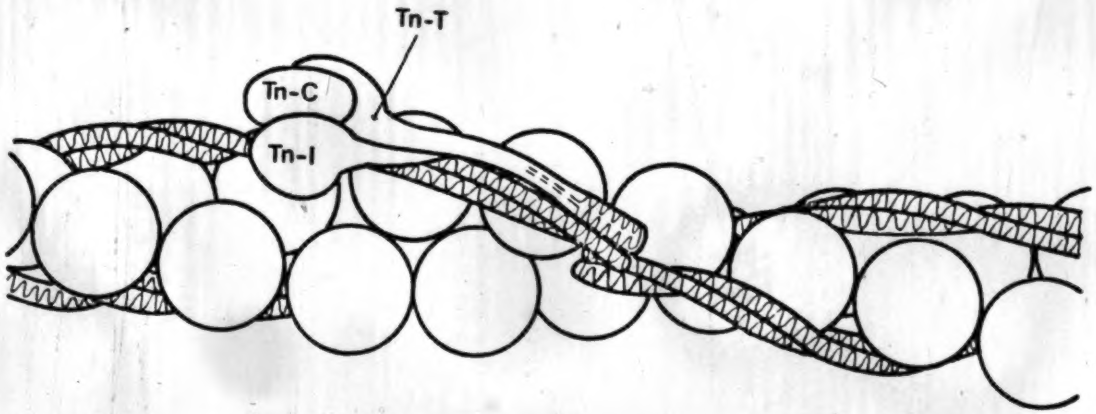


Fig. 1.7 A model for the molecular arrangement of the thin filament proteins. Two strands of actin molecules (open circles) form the core of the structure. Tropomyosin molecules are illustrated as coiled-coils which lie in the grooves of the actin filament. The troponin complex (TnI, TnC, and TnT) interacts with the COOH-terminal of the tropomyosin molecules. Each tropomyosin spans 7 actin molecules and is associated with one troponin complex. Tropomyosin and troponin form the basic regulatory unit of the thin filament. [Courtesy of Dr. L. Smillie]

a. ACTIN

Actin is the major component of the thin filament and can exist as a globular monomer (G-actin, M.W. 42,000) or a fibrous polymer (F-actin). In skeletal muscle all actin is in a filamentous form, which is composed of two strands of F-actin twisted helically about one another (Fig. 1.7). In vitro, a solution of G-actin can spontaneously aggregate into F-actin by increasing the ionic strength (2 mM Mg^{2+}, 50 mM KCl) in the presence of ATP. The polymerization process is probably driven by the hydrolysis of ATP and stabilized by the tighly bound divalent cation(Mg^{2+}) and ADP.

Actin has been sequenced by Collins and Elzinga (1975) and is found to be highly conserved in a variety of different cells and tissues (Vandekerckhove and Weber, 1978). Rabbit skeletal actin contains five cysteine residues, however cysteine 373 can be specifically labelled by thio-reagents in F-actin (Elzinga et al., 1973). Recently, Knight and Offer (1978) have identified cysteine 373 as being involved in the actin-actin interaction. F-actin can interact with myosin filaments as seen in Fig. 1.4b. This crossbridged complex is called actomyosin. F-actin can in fact significantly increase myosin ATPase activity. Therefore, the formation of crossbridges (actomyosin) is closely related to the hydrolysis of ATP and the generation of force for muscular contraction. This process will be discussed in greater detail later in this Chapter.

b. TROPOMYOSIN

Tropomyosin (TM) is composed of two α-helical polypeptides wrapped around one another in a coiled-coil fashion with a molecular weight of 66,000 and a length of 41 nm. In thin filaments tropomyosin molecules are polymerized in a head to tail fashion and lie in the grooves formed by the F-actin filaments (Fig. 1.7). Also, one tropomyosin molecule spans approximately seven actin molecules on each of the two strands and binds one troponin complex, thus providing a cooperative link throughout the thin filaments. Rabbit skeletal tropomyosin consists of α and β subunits in 4:1 molar ratio. In contrast rabbit cardiac tropomyosin contains only α-subunits. Both the α and β chains of tropomyosin have been sequenced by Smillie and his coworkers (for review see Smillie, 1979).

c. TROPONIN

Troponin (Tn) is localized with a periodicity of 385 Å on the thin filaments and is a complex of three polypeptide chains in 1:1:1 molar ratio. Each polypeptide chain has a special function in the regulation of muscle contraction. Rabbit skeletal troponin has a subunit which is capable of inhibiting the actomyosin ATPase in conjunction with tropomyosin.

This subunit is called troponin I (TnI, the inhibitory component) and the primary sequence has been reported by Wilkinson and Grand (1975). Troponin I has molecular weight of 21,000.

The calcium binding component of rabbit skeletal troponin (TnC) has four calcium binding sites and a molecular weight of 18,000. The primary sequence of TnC was determined by Collin et al., 1973. Two of the four calcium binding sites have a calcium binding affinity 10^7 M^{-1} and a magnesium binding affinity 10^3 M^{-1} and are known as the high affinity sites. The other two sites known as the low affinity sites are calcium specific with a calcium binding affinity 10^5 M^{-1}. When calcium is bound to these two calcium specific binding sites, TnC undergoes distinct conformational changes and can reverse the troponin I inhibition of the actomyosin ATPase (for review see McCubbin and Kay, 1980).

The last component of troponin complex, troponin T, (TnT, tropomyosin binding subunit) is an asymmetrical protein which is able to bind to tropomyosin. Its primary structure has been determined (Pearlstone et al., 1976) and it has a molecular weight of 30500. With the interaction of three troponin subunits with each other and TnT binding the complex to tropomyosin the regulatory complex (TM-Tn) is formed.

C. The Relationship Between Calcium Concentration And The Generation Of Force

How is muscle contraction triggered by the incoming nerve impulse? It is now generally accepted that the nerve impulse depolarizes the sarcolemma which in turn transmits the message via the T-tubes to the sarcoplasmic recticulum, causing it to increase in permeability. As a result, Ca^{2+} ions escape from the cisternae (a calcium ion storage vesicle or compartment of the sarcoplasmic recticulum) into the sarcoplasm. This extremely rapid discharge of calcium ion raises the concentration of calcium ion in the sarcoplasm from 10^{-7} M to 10^{-5} M. At this concentration, the low affinity calcium-binding sites of troponin C are filled, and troponin C undergoes a conformational change. This conformation change is transmitted to the other members of the regulatory complex (TM-Tn) such that the inhibition of the fibrillar ATPase is released and the formation of crossbridges can occur. The force is generated from the hydrolysis of ATP and the thick and thin filaments sliding past each other (see Fig. 1.8). The overall effect is muscle contraction.

When the nerve impulses cease, calcium ions are taken up by the sarcoplasmic recticulum membrane bound calcium-binding protein, called calsequestrin. Also, an ATP-dependent calcium pump active-transport system in the cisternae transports calcium from the sarcoplasm back into the cisternae against a calcium concentration gradient at

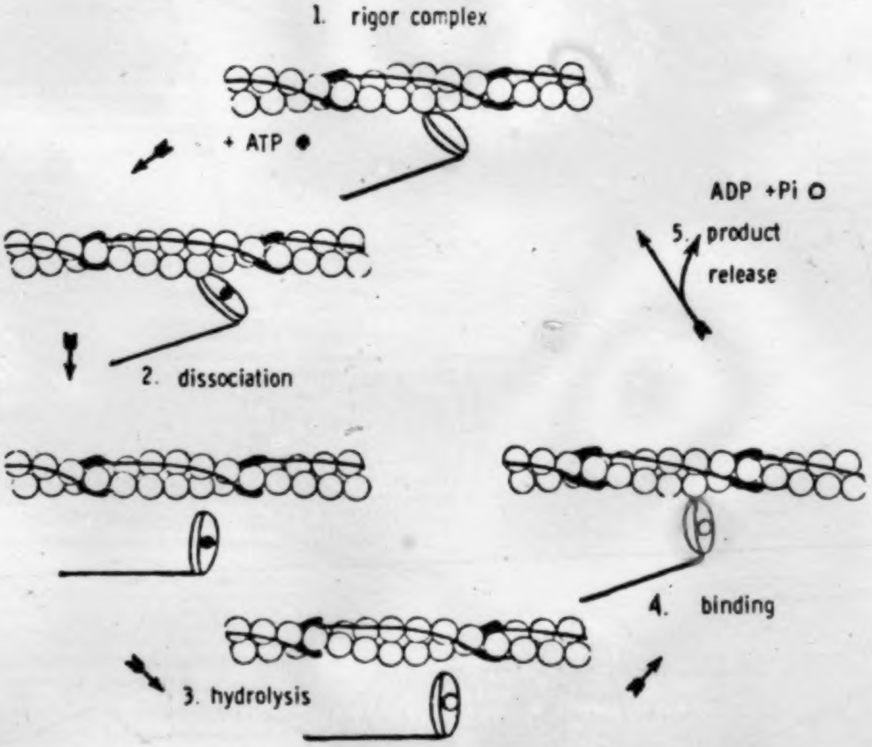

1. rigor complex
+ ATP
ADP +Pi
5. product release
2. dissociation
4. binding
3. hydrolysis

Fig. 1.8. Model for the Actomyosin ATPase Cycle.

(1) Nucleotide free myosin binds strongly as a rigor complex to F-actin. It has 45° binding angle relative to the thin filament.

(2) The binding of ATP to myosin weakens the interaction with actin and results in dissociation. The myosin head reverts back to a 90° state (binding angle).

(3) Myosin hydrolyses ATP to ADP.Pi. It now regains its high affinity for actin.

(4) Binding to actin facilitates the release of products.

(5) As product release occurs, the myosin head goes from 90° state to 45° state. This change in angle represents the Power Stroke.

the expense of the free-energy of hydrolysis of ATP. As soon as calcium concentration reduces to 10^{-7} M, TnC undergoes the reverse conformational changes such that the regulatory complex (tropomyosin-troponin) prevents further ATP hydrolysis and the sarcomeres return to their relaxation state. The source of energy for muscle contraction is ATP which is regenerated from ADP and phosphocreatine as well as by other metabolic processes.

Phosphocreatine + ADP ---------> Creatine + ATP

D. Current Models of Actin-linked Regulation of Muscle Contraction

In the previous section, I mentioned the principle role of calcium in the regulation of the contraction cycle of skeletal muscle, but the detailed molecular mechanism of how the calcium induced conformational change in troponin C is transmitted through the other regulatory components to all actin molecules in the thin filaments to activate the actomyosin ATPase remained to be solved.

1. Steric Blocking Model

In 1970, Moore et al., obtained the first three-dimensional reconstructions from electron micrographs of negatively stained actin filaments decorated with S-1 head of myosin. The observed tropomyosin shift from X-ray diffraction and electron microscopy studies was combined with the postulated myosin attachment position to produce the so-called steric blocking model of thin filament regulation (Huxley, 1972; Haselgrove, 1972; Perry and Squire,1973; Wakabyashi et al., 1975). This model indicated that the "off" position for tropomyosin is very close to the attachment site of myosin to actin, but the "on" position is clear of this site (Fig. 1.9 a&b). Therefore tropomyosin in the "off" position could physically block the attachment of myosin to actin in the relaxed muscle, but would roll into the groove and out of the way of the attachment site when calcium binds to troponin. On the basis of the results

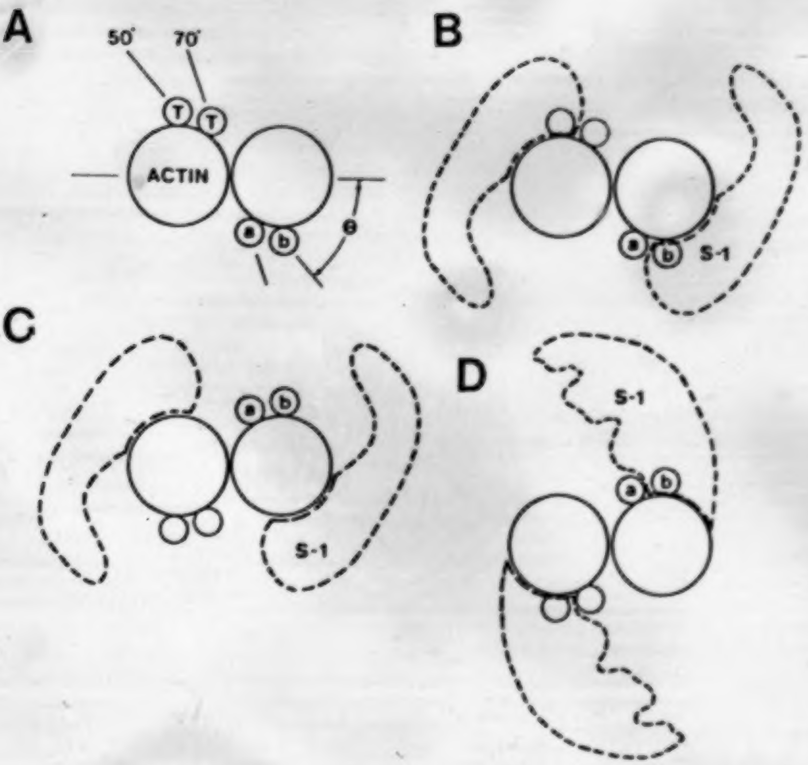


Fig. 1.9 Schematic illustrations of the proposed models of regulation.

(A) and (B) are the Steric Blocking model proposed by Huxley (1972). The myosin head (S1) attachment is close to TM position "b" (the blocking position), but it is away from position "a" (the active position). (C) is the model proposed by Seymour and O'Brien (1980) where TM is positioned opposite to the myosin head position. Therefore the steric blocking model could not be used to explain the regulation mechanism. (D) is the latest model proposed by Taylor and Amos (1981). Their observations agreed with those of Seymour and O'brien as far as the position of TM on the actin filament however they showed the myosin head to be on the same side of TM. Thus the steric blocking model is still valid.

obtained from the myofibril ATPase activity of reconstituted filaments from various components of troponin, tropomyosin, actin and myosin S-1, this blocking model was further modified (Hitchcock et al., 1973; Potter and Gergely, 1974; see Fig. 1.10). Moreover, Potter and Gergely (1974) suggested that the troponin complex was always anchored to F-actin-tropomyosin filaments via troponin T in the presence or absence of calcium. Troponin I in the absence of calcium was bound either to actin or to a site made up of actin and tropomyosin in such a way as to block the myosin interaction with actin (Fig. 1.10a). This TnI-F-actin-tropomyosin binding was broken in the presence of calcium, so that myosin head and actin were free to interact (Fig. 1.10b). Recent photochemical cross-linking studies on reconstituted thin filament by Sutoh and Matsuzaki (1980) indicated that cross-linking between TnI and actin occured in both the presence and absence of Ca^{2+}. However, in the presence of Ca^{2+} the percentage of TnI-actin cross-linking is decreased while TnC-TnI cross-linking is increased. They also found that no cross-linking could be detected between TnI and tropomyosin. These results imply that i) TnI is also involved in maintaining troponin complex binding to the thin filament in the presence of Ca^{2+}, ii) the binding affinity of TnI for TnC is much stronger than that for actin in the presence of Ca^{2+} such that TnI-TnC complex could be dissociated from F-actin as seen under the conditions used by Potter and Gergely (1974) in the sedimentation

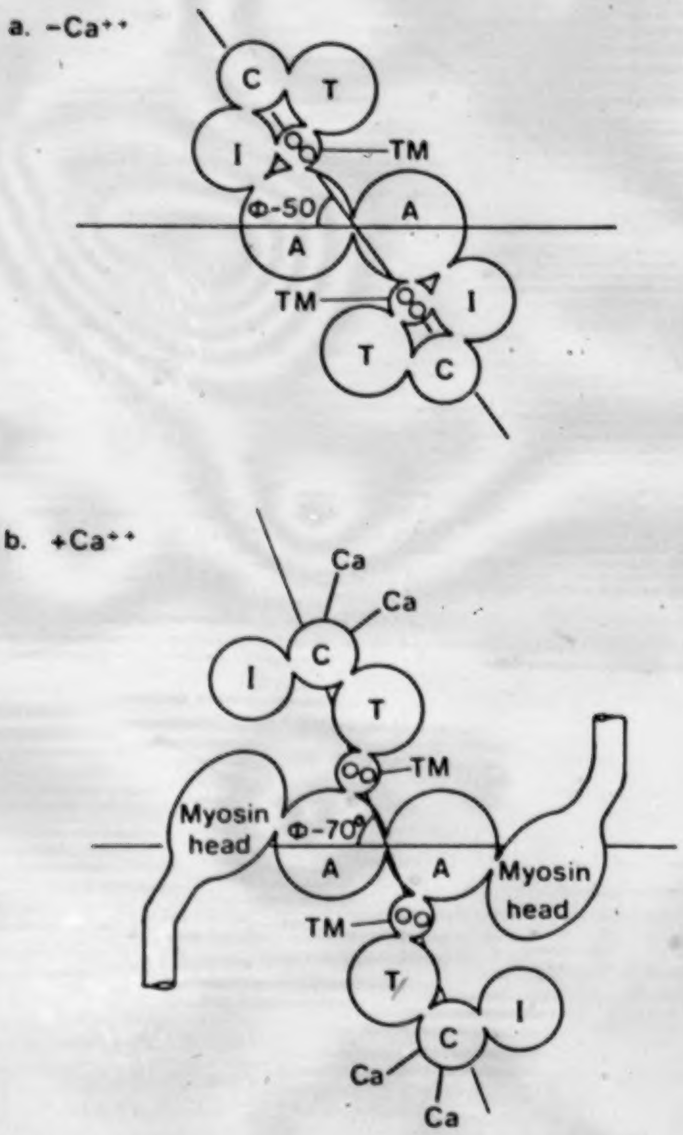

a. −Ca⁺⁺
C
T
I
TM
Φ-50
A
A
TM
I
T
C
b. +Ca⁺⁺
Ca
Ca
C
I
T
TM
Myosin head
Φ-70°
A
A
Myosin head
TM
T
C
I
Ca
Ca

Fig. 1.10 The Steric Blocking Model

(a) In the absence of Ca^{2+}, TnI binds to actin and anchors the TM to the periphery of the actin grooves. In this position TM is postulated to physically block the binding of the myosin head.

(b) In the presence of Calcium, a conformational change in the Tn complex is transmitted to TM resulting in a movement closer into the F-actin grooves which opens up the myosin binding sites. [From McCubbin and Kay, 1980] experiments. Thus, the steric blocking model proposed by Potter and Gergely (1974) might require modification.

Before 1980 there was no solid evidence that the myosin head binding site and tropomyosin strands were on the same side of the actin groove. The steric blocking model was postulated as shown in Fig. 9 a&b. Seymour and O'Brien (1980) using X-ray diffraction and the three-dimensional image reconstruction of electron micrographs of thin filaments and pure F-actin filaments decorated with myosin S-1, showed that tropomyosin did not lie on the same side of the actin groove as the myosin-binding site suggested by Moore et al. (1970) (Fig. 1.9c). This result obviously opposed the steric blocking model. Before a new model could be proposed, Taylor and Amos (1981) reinvestigated the problem and found that tropomyosin and myosin attachment sites on actin were indeed close together (Fig. 1.9d). It is certain that three-dimensional reconstructions from X-ray diffraction pattern and electron micrographs need refinements.

2. Allosteric Blocking Model

Nobody assumed that the steric blocking model would prove to be the whole story. Not only because there is controversy as to where tropomyosin strands lie on the actin groove, but the steric blocking model also does not explain the well-documented biochemical evidence that the presence of tropomyosin alone can potentiate the actin activation of the Mg^{2+}-ATPase of the myosin head at low ATP concentration (Bremel et al., 1972; Weber and Murray, 1973; Eaton et al., 1975; Eaton, 1976). In terms of the steric blocking model, these observations imply that when tropomyosin moves from the relaxed "off" state to the contracted "on" state, it not only releases the blocked actin binding site for myosin but also enhances the actomyosin interaction in a way that increases the Mg^{2+}-ATPase activity above that of actomyosin alone. There are two ways to explain the tropomyosin potentiation. Firstly, the potentiation could result from a direct interaction between tropomyosin and the myosin head. this seems unlikely because tropomyosin does not bind to myosin in the absence of actin (Kaminz and Meruyama, 1967). The second possibilty was proposed by Weber and Murray(1973) who account for the potentiation in terms of a cooperative effect in which actomyosin formation induces a conformational change in the associated actin monomer, and tropomyosin molecules then transmit an appropriate signal to the other actin monomers associated with them so that myosin binding is increased. Whether or not this potentiation has to do with the position of tropomyosin on the F-actin groove remains to be seen. However, recent studies (Chalovich et al., 1981; Chalovich and Eisenberg, 1982) suggest that troponin-tropomyosin does not sterically block the binding of myosin S-1-ATP and S-1-ADP-Pi to actin (Fig. 1.8, step 4). The regulatory complex (troponin-tropomyosin) may some how interfere with the rotation of the myosin head on the actin, thus increasing the activation energy for this step and decreasing the rate of Pi release and the ATPase activity. This interference may be an allosteric effect or an indirect conformational change in the actin as proposed by Weber and Murray (1973). What is clear at the present time is that thin filament regulation is by no means as simple as the steric blocking model.

E. Molecular Structure of Thin Filament

Though a great deal has been learned about thin filament regulation, it is essential to know the detailed molecular structure of the thin filament components as well as their spatial orientation in order to understand fully the regulation process. The following sections are a general survey of what is presently known about the sites of interaction between components of the thin filament.

1. Tropomyosin and Actin Interaction

Eaton et al.(1975) showed that ^{125}I-labelled tropomyosin has very low binding to F-actin in 1 mM Mg^{2+} , 2 mM ATP, 30 mM KCl buffer, pH7.0. However, the maximal binding can be achieved by either increasing KCl concentration to 100 mM or Mg^{2+} and ATP to 5 mM and 3 mM respectively. These results indicate tropomyosin binds very well to F-actin under physiological conditions.

The sites of interaction between tropomyosin and actin have not been studied extensively. Yet two recent reports using SH-directed fluorogenic reagents (Tawada et al;, 1978; Tao and Cho, 1979) indicate that tropomyosin binds to actin at a site near cysteine 373. Also, a recent preliminary report by Johnson et al.(1981) has shown that tropomyosin alone (i.e in the presence or absence troponin and calcium ions) can protect arginine 95 of actin from chemical modification by 1,2-cyclohexanedione. They suggest that the region around arginine 95 is involved in the attachment of tropomyosin to actin in both the "on" and "off" states suggesting that tropomyosin does not move during the contraction process. Moreover, a report by Lin and Dowhen (1980) has provided additional evidence from fluorescence energy transfer studies between chromophores on actin and tropomyosin that there is no appreciable movement of tropomyosin across the actin molecule as a result of calcium binding to troponin. These results oppose the steric blocking model.

So far, there is no direct evidence indicating the sites of interaction of actin on tropomyosin. Recently, Smillie's group has accumulated evidence which indicates that the head-to-tail polymerization of tropomyosin molecules (involving an 8 to 9 amino acid residues overlap between the COOH- and NH_2-terminal ends of adjacent molecules) is of crucial importance for the assembly and function of the thin filament protein complex (Mak and Smillie, 1981; Cote et al., 1978a and b; Cote and Smillie, 1981). By averaging the α-helix parameter of Chou and Fasman (1974) over extended segments of the tropomyosin sequence, Smillie et al.(1981) predict: 1) The well developed periodicity of the α-helix parameter in the central and NH_2-terminal half of the molecule interacts more strongly and in a more regular fashion with actin monomers. 2) The COOH-terminal half, on the other hand, may be more loosely associated with the actin molecule and be more subject to conformational changes through interaction with the troponin complex.

2. Tropomyosin and Troponin Interaction

Evidence for the binding site on TM for Tn is derived largely from electron microscopic examination of crystals and fibrous magnesium tactoids of TM in the presence and absence of Tn and its components. These studies have suggested that the site of Tn-binding is about one third of the distance from the COOH-terminal end of TM(Nonomura et al., 1968; Cohen et al.,1971 a and b; Hitchcock et al.,1973; Margossian and Cohen,1973; Ohtsuki,1974; Stewart and McLachlan,1976; McLachlan and Stewart,1976). Recent evidence has indicated that two regions of TnT may be involved in the binding of the troponin complex to tropomyosin. From a chymotryptic digest of TnT, fragments T1 and T2 were isolated and identified as residues 1-158 and 159-259, respectively, both of which were bound to TM immobilized on a Sepharose affinity column (Ohtsuki, 1979; Katayama,1979; Pearlstone and Smillie, 1981). The chymotryptic fragments T2' (residues 159-227) and T3 (residues 228-259) showed no interaction with immobilized TM, while a BNPS-skatole cleavage fragment of TnT (residues 206-258) showed a weak interaction (Pearlstone and Smillie,1981). These results suggested that the binding region represented by T2 extends over a relatively large portion of the COOH-terminus of TnT. Furthermore, the immunoelectron microscopic observations reported by Ohtsuki(1979,1980) showed that the position of antibody against T2 on the thin filament was the same as the position of antibodies against TnC and TnI; that is, one-third of the distance from the COOH-terminus of TM in the vicinity of Cys-190. Antibody against T1 was situated about 13 nm from the antitroponin-T2 site in the direction of the COOH-terminus of TM. In agreement with these observations Mak and Smillie(1981) have demonstrated that nonpolymerizable TM (11 residues removed from the COOH-terminal end of TM with carboxypeptidase A) no longer binds to the CB1 fragment (residues 1-151) of TnT and that the extent of iodination of tyrosine residues 261 and 267 of TM is markedly reduced in the presence of CB1. These observations all suggest that T1 (or CB1) binds close to or at the COOH-terminal end of the TM molecule and may perhaps involve the head-to-tail overlap region of TM molecules. The suggestion by Nagano et al.(1980) and by Ohtsuki(1980), based solely on predictive methods, that residues 90-148 of TnT interact with residues 148-214 of TM seem inconsistent with the above experimental facts.

Based on the fact that T2 binds to both immobilized TM and nonpolymerizable TM and that this region of TnT interacts with both TnC and TnI (Ohtsuki,1979; Katayama,1979; Pearlstone and Smillie,1978 and 1980), it has been postulated that this fragment binds in the region of cysteine 190 of TM and that this binding would be calcium sensitive in the presence of TnC(Mak and Smillie,1981). This structural interpretation of the two-site binding of troponin on tropomyosin is supported by the demonstration of Morris and Lehrer(1981) that the fluorescence of TM labeled with N-(1-anilinonaphthyl-4) maleimide at cysteine 190 is enhanced by the addition of T2 but unaffected by the presence of T1. This suggests that T2 is in close proximity to cysteine 190 and is consistent with the conclusion that T1 (or CB1) is bound close to the COOH-terminal end of the TM molecule.

3. Interaction Between Troponin Subunits

To understand the control mechanism of the Ca^{2+}-induced conformational changes in the troponin complex, many studies involving a variety of chemical, physical, and structural techniques have been carried out to demonstrate the interactions between the binary complexes, TnI-TnC and TnC-TnT (see reviews Perry, 1979; Ebashi, 1980; McCubbin and Kay, 1980). It was only recently that TnI-TnT interaction was clearly established by a variety of techniques: cross-linking reagents (Hitchcock, 1975; Sutoh and Matsuzaki, 1980), circular dichroism, ultraviolet absorption difference spectroscopy, and gel-filtration (Horwitz et al., 1979; Hincke et al., 1979; Pearlstone and Smillie, 1980), and affinity chromatography (Katayama, 1979; Reisler et al., 1980). However, there still exists disagreement in the relative importance of these interactions. Ebashi(1980) considers the TnC-TnT interaction not essential with the important interactions occuring between TnI-TnC and TnI-TnT in the troponin complex. In order to clarify our understanding of the Ca^{2+}-induced conformational changes in the troponin complex, it is essential to identify the sites of interaction at the molecular level in troponin in the presence and absence of calcium. In this regard, numerous studies have been attempted to determine the sites of interaction of the various components of the troponin complex. These results are summarized in Table 1-II.

One thing we should keep in mind in interpretating the results obtained from the studies of reconstituted binary complexes as seen in Table 1-II. Do the binding properties of a fragment of one protein or even the whole protein itself with another protein really tell you anything about how that binary complex interacts in a multi-subunit system? Certainly, interpretation of the results obtained with the two component systems must be made with reservations since the thin filament consists of five different interacting proteins. Nevertheless, as seen in Table 1-II a number of points emerge about the troponin complex that may be of relevance to its mechanism of function in the regulation of muscle contraction.

i) All three troponin subunits (TnC, TnI, and TnT) interact with each other. TnT links the troponin complex to tropomyosin (Perry, 1979).

ii) The interaction of troponin C with troponin I and troponin T may involve two or more sites on each of the components involved (Table 1-II). Also, troponin I and

TABLE 1-II

The Regions of the Individual Subunits of Troponin Complex involved in the Sites of Interaction

Reconstituted Binary Complex	Residue Regions of protein involved in sites of interaction			Reference
	TnC	TnI	TnT	
TnC-TnI	84-135	5-27		a
		98-116[1]		a
TnC-TnT	84-90			b
			206-258[2]	c,d
			159-209[3]	c
TnI-TnT		around 48, 64, 133[4]		e,f
			11-43 159-209	g

Reference: a) Perry(1979) b) Hitchcock(1981)
c) Pearlstone and Smillie(1978)
d) Ohara et al.,(1980)
e) Horowitz et al.,(1979)
f) Hincke et al.,(1979)
g) Pearlstone and Smillie(1980)

[1] This region (residue 98-116) of TnI is able to inhibit actomyosin ATPase activity in the presence of tropomyosin.

[2] This region (residue 206-258) of TnT binds to TnC much stronger in the presence of calcium, and also binds to tropomyosin (Pearlstone and Smillie, 1981).

[3] This region (residue 159-209) of TnT also binds to TnI.

[4] The oxidized TnI will not bind to TnT, thus cysteine residues 48, 64, and 133 regions of TnI may be involved in the sites of interaction with TnT.

troponin T interactions may involve two sites on each of the components (Table 1-II).

iii) The region around residue 206-258 of troponin T interacts with troponin C as well as tropomyosin (Pearlstone and Smillie, 1981).

iv) The region around residue 98-116 of troponin I binds to both troponin C and actin-tropomyosin complex (Perry, 1979).

v) The region around residue 159-209 of troponin T binds to both troponin I and troponin C (Pearlstone and Smillie, 1980).

vi) The interaction between troponin I (residue 98-116) and troponin C (residue 84-135) is probably largely electrostatic in character (Perry, 1979).

vii) Phosphorylation sites on troponin I and troponin T are in all cases very close to the interaction sites with troponin C (Perry, 1979).

If all these binding sites really do exist in the native thin filament, one can easily realize that a relatively slight movement of the troponin C molecule upon calcium binding will change profoundly the interaction occuring in the thin filament.

F. Purpose of This Study

As discussed in the "Molecular Structure of Thin Filament" section, a great deal of work has been carried out to identify the sites of interaction between the components of the thin filament. All these results still do not provide the spatial orientation of the binding sites. X-ray crystallographic data of actin, tropomyosin, native troponin complex, and the individual troponin subunits are not available. Therefore we think the best approach to obtain spatial information would be to attach a bifunctional cross-linking reagent specifically to a known position in one component of the thin filament at a 1:1 molar ratio, followed by cross-linking and sequence analysis of the site attachment of the cross-linker. The results from the sequence analysis of the inter and intra cross-linking would provide the spatial orientation of each component in the filament with respect to the site of attachment. Furthermore, by comparing the cross-linking results in the presence and absence of calcium, one should be able to examine the influence of calcium on the conformational changes occuring in the native thin filament. Therefore, the first aim of my project was to design and synthesize a heterobifunctional cross-linking reagent, and then to demonstrate the feasibility of using such a probe to study protein-protein interactions in a multi-subunit system such as the thin filament.

TABLE 1-III

Sulfhydryl (SH) Groups Supected in the Proximity of the Sites of Interaction between Components in the Rabbit Skeletal Muscle Contraction System

Protein Complex	Cysteine residue position in protein	Reference
TnC-TnI	98 of TnC	a
TnI-TnT	48 and 64 of TnI	b
Tn-TM	190 of TM	c
Tn-TM-Actin	133 of TnI	d
TM-Actin	373 of Actin	e
Actin-Actin	373 of Actin	f
Actin-Myosin	373 of Actin	g
Myosin ATPase Catalytic site	SH1 and SH2 of Myosin S-1	h

REFERENCE: a) Perry (1979)
b) This study (Chapter III)
c) McLachlan and Stewart (1976)
d) Ohyashiki and Sekine (1979) and This study (Chapter III)
e) Tao and Cho (1979)
f) Knight and Offer (1978)
g) Knight and Offer (1980)
h) Ramirez et al., (1979) and Wells and Yount (1979)

We have chosen the sulfhdryl (SH) groups for the sites of specific attachment because SH groups, present in limited numbers in proteins, have been shown to be functionally important and near to the sites of protein-protein interactions. More specifically, in the rabbit skeletal muscle contraction system, SH groups are suspected in the proximity of the sites of interaction between troponin C and troponin I (Perry, 1979), troponin and tropomyosin (McLachlan and Stewart, 1976), actin-actin (Knight and Offer, 1978), tropomyosin and actin (Tao and Cho, 1979; Tawada et al., 1978),troponin I and troponin T (Horwitz et al., 1979; Hincke et al., 1979), actin and myosin (Knight and Offer, 1980), troponin-tropomyosin-actin (Ohyashiki and Sekine, 1979), and at the ATPase catalytic site on myosin S-1 (Ramirez et al., 1979; Wells and Yount, 1979) (Table 1-III). Therefore, a bifunctional cross-linking reagent that could be specifically attached to these SH groups would be invaluable for the identification of the proteins in the vicinity of the labelled SH group and enable the determination at the molecular level of the amino acid residues involved in the sites of interaction or the catalytic mechanism of myosin ATPase.

We feel that the other end of the heterobifunctional cross-linker should be a nonspecific cross-linking functional group, so that the efficiency of cross-linking formation would not be affected by pH, temperature, and the availability of suitable reactive groups at the sites of interaction. The ideal nonspecific cross-linking functional group is the aryl azide, which is chemically inert, but photo labile. Its photoactivated species (nitrenes) reacts indiscriminately with whatever chemical groups they encounter in the binding site. This should increase the efficiency of formation of cross-linking (for review see Bayley and Knowles, 1977; and Chapter IV). With an aryl azide as the cross-linker and the SH group for the site specific attachment, we have designed, synthesized and characterized a new heterobifunctional photoaffinity probe, N-(4-azidobenzoylglycyl)-S-(2-thiopyridyl)cysteine (AGTC). Its chemical and physical properties will be discussed in Chapter IV.

Besides working on the organic synthesis of the photoaffinity probe, I have also investigated the topographical relationship of the SH groups of rabbit skeletal troponin to provide information for the specific labelling of SH groups with our heterobifunctional photoaffinity probe (AGTC) or other probes for the purpose of determining the sites of interaction and for monitoring the calcium-induced conformational changes in troponin (see Chapter III).

In order to demonstrate the general utility and the feasibility of using our heterobifunctional photoaffinity probe (AGTC) for studying protein-protein interactions, and to throw some light on the control mechanism of the Ca^{2+}-induced conformational changes in the regulatory protein complex (troponin-tropomyosin), we choose to study the interaction involving troponin C and troponin I (Chapter V), the interaction between tropomyosin and troponin (Chapter VI), and the interactions within the troponin complex (Chapter VII).

If a crosslinking reagent is attached on a residue at the site of interaction between two proteins, it must be kept in mind that the crosslinking reagent could distort the local conformation or even prevent the interaction. Therefore, control experiments must be carried out to ensure that the modified protein (crosslinker attached) has not caused any significant changes in the integrity of the complex. These experiments should include the tests of complex formation, biological activity and structural changes. Also after having identified the crosslinking site one still must examine the amino acid sequence of the regions involved in order to postulate the binding regions and residues involved (Chapter VII).

II. GENERAL MATERIALS AND METHODS

A. Sources of Materials

1. Chemicals And Solvents

Tris(ultra pure), phenylmethylsulfonyl fluoride(PMSF), diisopropyl phosphofluoridate(DFP) and cyanogen bromide(CNBr) were purchased from Sigma Chemical Company, Inc., and used without further purification. Iodoacetamide, and 2,2'-dithiopyridine were obtained from Aldrich Chemical Company, Inc. [1-^{14}C]-Iodoacetamide and [2-^{3}H]-Glycine were obtained from New England Nuclear. [2-^{14}C]-p-aminobenzoic acid was purchased from ICN Company. Ammonium sulfate(ultra pure) and urea(ultra pure) were products from Mann Research Laboratories. Dithiothreitol(DTT) was purchased from Bio-Rad Laboratories Ltd. All other chemicals and organic solvents such as methanol, acetone, ether, etc, were reagent grade and obtained from Fisher Chemical Company, Inc.

2. Resins

Sephadex G-25 (superfine), G-50(superfine), G-100(medium), DEAE-A50, Sephacryl S-200(40-105 um), and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P200, Hydroxylapatite and Affi-Gel Blue were purchased from Bio-Rad Laboratories(Canada) Ltd., Mississauga, Ontario.

3. Enzymes

TLCK-Chymotrypsin and TPCK-Trypsin were purchased from Sigma Chemical Company and Worthington Biochemical Company respectively.

4. Muscle Tissues

Frozen rabbit skeletal and cardiac muscle (type I, mature, New Zealand white) were purchased from Pel-Freeze Biologicals. Fresh beef hearts were obtained from Swift, Edmonton.

B. Methods

1. Protein Purification

a. TROPONIN

Skeletal and cardiac troponin were prepared essentially as described by Staprans et al.(1972) with the following modifications. Centrifugation at 4200 rpm for 10 min in an IEC-DPR-6000 centrifuge was used instead of the extraction through cheese-cloth. The final pH 4.5 precipitation was repeated, after which the supernatant was adjusted to pH 7.6 with 1M Tris and an $(NH_4)_2SO_4$ fraction was carried out by the addition of solid $(NH_4)_2SO_4$. Precipitated residue was collected at 40-55% $(NH_4)_2SO_4$ at 4°C by centrifugation at 4200 rpm for 25 min in an IEC-DPR-6000 centifuge. The precipitate was dissolved in distilled water, dialyzed against 4x20 litres of 2 mM β-mercaptoethanol and 2mM NH_4HCO_3, and then lyophilized. Troponin was stored as a lyophilized powder at -20°C.

Rabbit skeletal troponin was further purified by the method of Reisler et al.(1980) with the following modifications. The crude troponin (600 mg) was dissolved in 60 ml of 0.25M KCl, 10 mM Tris-HCl, 1 mM DTT buffer, pH7.0 and dialyzed at 4°C overnight against 1 litre of the same buffer. The protein solution was clarified by centrifugation (20,000 rpm) for 10 min. The supernatant was loaded onto a 2.5 x 50 cm Affi-Gel blue column operated at 4°C. The column was washed with two column volumes of above starting buffer and the purified troponin was eluted with 0.7M KCl, 10 mM Tris-HCl, 1 mM dithiothreitol buffer, pH7.0 at a flow rate 60 ml/hr. The purified troponin was collected, dialyzed against 2 mM NH_4HCO_3, 2 mM β-mercaptoethanol solution, lyophilized, and was stored at -20°C. A sample elution profile is shown in Fig. 2.1.

b. TROPONIN SUBUNITS(TnI, TnT, and TnC)

Troponin was separated into its subunits(TnI, TnT, and TnC) as described by the method of Greaser and Gergely(1973) using DEAE A-50 Sephadex chromatography in the presence of 50 mM Tris-HCl, 1 mM EGTA, 1mM DTT, 8M urea buffer, pH7.8. To reduce the possibility of proteolysis, the troponin was usually pretreated with 5% formic acid for 1 hr. The chromatographic conditions are described in the Chromatography section that follows in this Chapter.

Bovine cardiac TnI was further purified by gel filtration on a Bio-Gel P200 column(2.5x100cm) as described by Burtnick et al.(1975).

c. TROPOMYOSIN

α-Tropomyosin was prepared in two stages. The first stage was the preparation of rabbit cardiac muscle powder according to the method of Bailey(1948) with the following modifications. Acetone was used instead of ether in the final step. The muscle powder was air dried

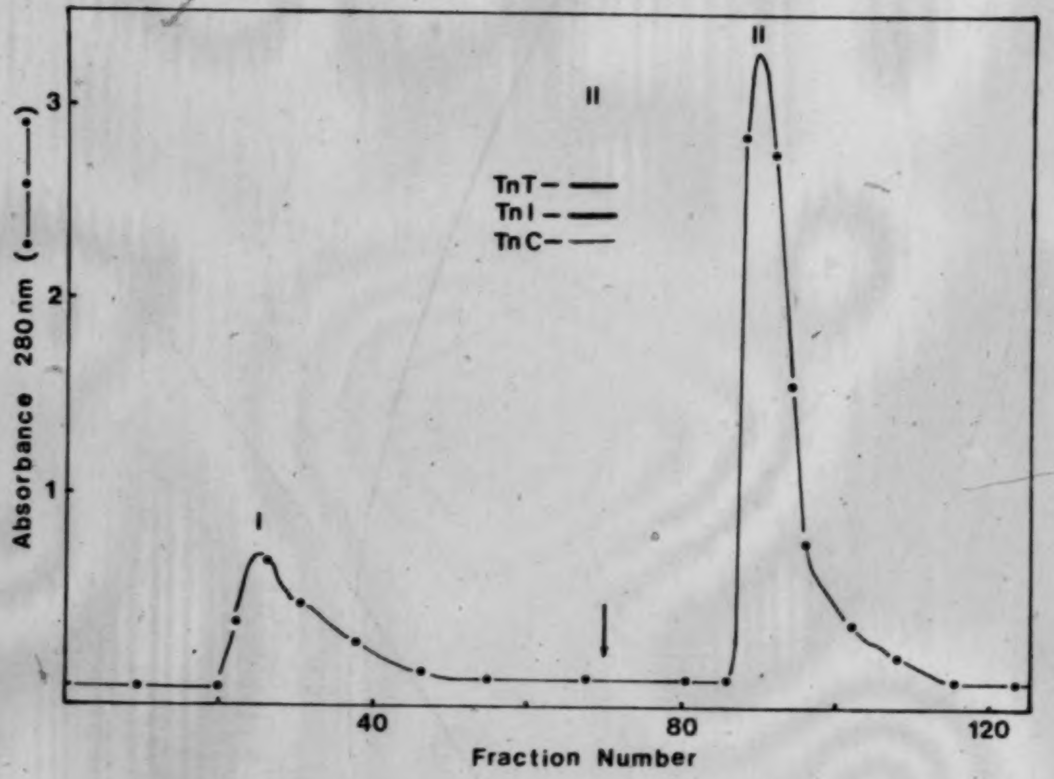


Fig. 2.1 Purification of rabbit skeletal troponin on Affi-Gel Blue column. The conditions of chromatography have been described in the "General Method". The arrow indicated the start of the 0.7 M KCl, 10 mM Tris-HCl, 1 mM DTT buffer, pH 7.0. Inset: SDS-Urea Laemmli gel electrophoresis of peak II.

and stored at -20°C.

Extraction of tropomyosin from the muscle powder was carried out as described by Pato et al.(1981). α-Tropomyosin was obtained in the 55-65% $(NH_4)_2SO_4$ cut and was further purified by hydroxylapatite chromatography as described by Eisenberg and Kielley(1974) to remove any contaminating troponin.

d. ACTIN

Rabbit skeletal actin was prepared in two stages. Actin acetone powder was prepared by the method of Carston and Mommnerts(1963), except that ethanol extraction (3 volumes) was performed before the acetone extraction. The acetone powder was stored at -20°C and appeared to be stable indefinitely.

The procedure of Spudich and Watt(1971) was used to prepare actin from the acetone powder. The only modification was that the extract containing actin was separated from the muscle residue by filtration through a thick paper pulp(Whatman #1 paper). The filtrate was always clear at this stage. The final purified G-actin (about 5 mg/ml) was stored at 4°C and used within a week of preparation.

2. Protein Concentration Determinations

a. ABSORBANCE MEASUREMENTS

Protein concentrations of pure samples were usually determined by means of the absorbance at 280 nm as given in the literature(Table 2-I).

b. AMINO ACID ANALYSIS

Amino acid analyses were always used to determine protein or peptide concentration. These were done in a Durrum model D-500 analyser. Samples of protein or peptide were hydrolysed in evacuated, sealed tubes with constant boiling HCl(5.7N) and 0.1% phenol for 24 hr at 110°C. The seal was broken and the hydrolyzate was dried down in a desiceator over NaOH pellets. The residues were redissolved in a known amount of pH2.2 buffer and aliquots were loaded on the analyser. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolyzate was used to calculate the concentration of protein or peptide. The color value used for S-carboxymethylated cysteine was 0.905 times the value for aspartic acid as described by Hodges(1971).

3. Chromatography

a. DEAE A-50 SEPHADEX CHROMATOGRAPHY

The DEAE A-50 Sephadex chromatographic method of Greaser and Gergely (1973) was used to separate and isolate troponin subunits(TnI, TnT, and TnC). Purity and yield have been improved by the following modifications. To reduce the possibility of proteolysis, crude or purified troponin (150 mg) was first treated with 5% formic acid for 1 hr and then lyophilized. The acid

TABLE 2-I

Protein Extinction Coefficients and Molecular Weights

Proteins	1 mg/ml Absorbance at 280 nm	Molecular Weights
G-actin	1.11 (a)	42,000 (b)
Rabbit Skeletal Troponin	0.47 (c)	70,000 (l)
TnC	0.193 (d)	18,000 (e)
Rabbit Skeletal TnI	0.59 (d)	21,000 (f)
Bovine Cardiac TnI	0.37 (g)	23,000 (g)
TnT	0.50 (h)	30,500 (i)
Tropomyosin	0.33 (j)	33,000 (k)

a) Houk and Ue (1974) b) Elzinga et al., (1973)
c) Lovell and Winzor (1977)
d) Marogssian and Cohen (1973)
e) Collins et al., (1973)
f) Wilkinson and Grand (1975)
g) Burtnick et al., (1975)
h) Eisenberg and Kielly (1974)
i) Pearlstone et al., (1977)
j) Cote' (1980) k) Stone et al., (1974)
l) sum of three subunits

treated troponin was dissolved into 8 ml of 8M urea, 50mM Tris-HCl, 1mM DTT, 1mM EGTA buffer, pH7.8 and then dialyzed overnight against the same buffer(500ml) at 4°C. The protein solution was loaded onto a DEAE A-50 Sephadex column(1.6x40 cm) which was previously equilibrated with the urea starting buffer described above. The column was washed with 90 ml of the starting buffer before beginning a linear salt gradient consisting of 150 ml each of starting buffer and starting buffer + 0.6M KCl at a flow rate of 10 ml per hr (see Fig. 2.2). The protein peaks were pooled and dialyzed against 4x20 litres of 1 mM HCl + 2 mM β mercaptoethanol and then lyophilized. TnI, TnT, and TnC were stored at -20°C.

b. TM-SEPHAROSE AFFINITY CHROMATOGRAPHY

Cyanogen bromide activated Sepharose 4B(40g wet weight) was prepared as described by Syska et al.(1974) and suspended in 8 ml of 0.1M $NaHCO_3$, pH8.3. α-Tropomyosin (300 mg) in 20 ml of 0.1M $NaHCO_3$, pH8.3, was added to the cyanogen bromide activated resin. The reaction mixture was shaken in a plastic vial on a 105° rocking shaker at room temperature for 2 hr and 6 hr at 4°C. Aminoethanol was added to quench the reaction(4 ml of 12M solution to give a final concentration of 1M) and the reaction mixture was shaken overnight at 4°C. The resin was then washed with 200 ml cold distilled and

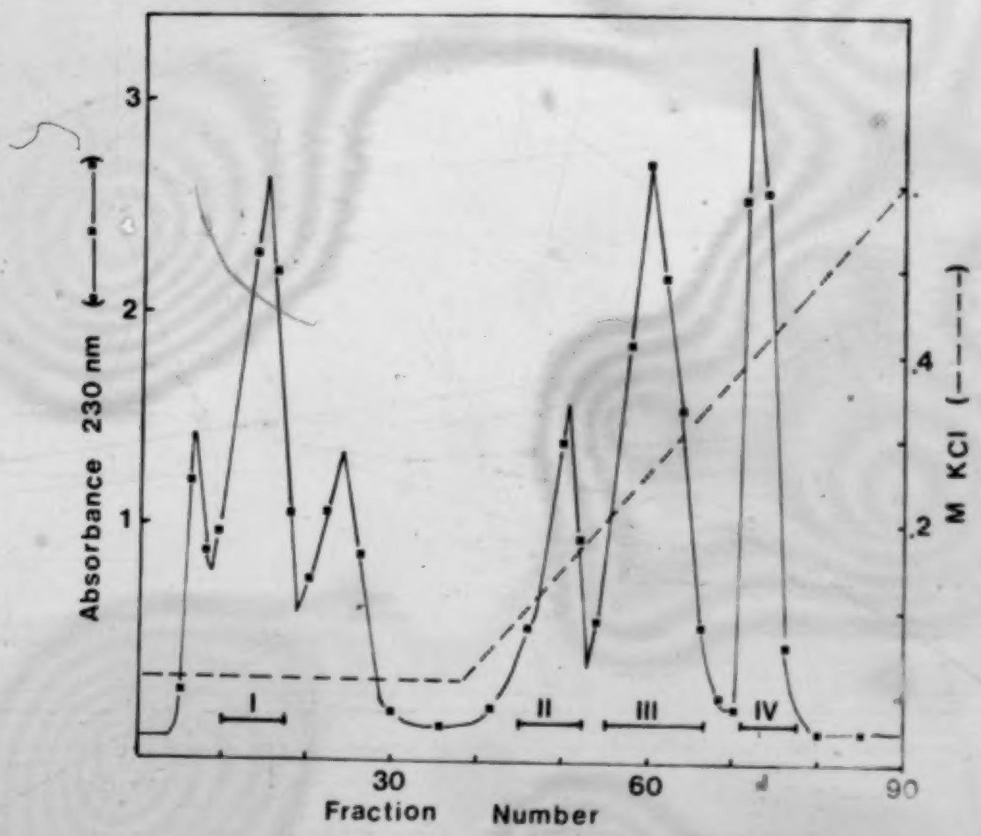


Fig. 2.2. Chromatographic Separation on DEAE A-50 Sephadex of Crude Rabbit Skeletal Troponin. The column(1.6 x 40 cm) equilibrated with 50 mM Tris-HCl, 8M urea, 1 mM EGTA, 1 mM DTT buffer, pH7.8, was eluted with a linear salt gradient (-----) from 0 to 0.6M KCl in the same buffer (150 ml each). Peaks I, III, and IV are TnI, TnT and TnC respectively.

deionized water followed by 200 ml of cold 0.1M $NaHCO_3$. The washing was repeated 4 times. Tm (137 mg) was recovered from the washes. The TM-Sepharose 4B was packed into a 1x40 cm column. The chromatographic conditions used are those described by Pearlstone and Smillie(1981).

c. HYDROXYLAPATITE CHROMATOGRAPHY

Hydroxylapatite chromatography was operated as described by Eisenberg and Kielly(1974) with the following modifications. Crude tropomyosin (120 mg) was dissolved into 12 ml of 10mM Na_2HPO_4, 1M KCl buffer, pH7.0, and then dialyzed for 8 hr against 1 litre of the same buffer at 4°C. The protein solution was applied to a hydroxylapatite column(1.0x40 cm), which was equilibrated with the above starting buffer. The column was eluted with 100 ml of the starting buffer at a flow rate of 10 ml per hr. Any contaminating troponin was eluted with 200 ml of 60 mM Na_2HPO_4, 1M KCl buffer, pH7.0. α-Tropomyosin was eluted with 300 mM Na_2HPO_4, 1M KCl buffer, pH7.0. The protein peaks were pooled, dialyzed against 2 mM NH_4HCO_3, lyophilized, and stored at -20°C.

d. GEL FILTRATION

All desalting experiments were done on a Sephadex G-25 column (1.5 x 100 cm) with either 1 mM HCl or 50 mM NH_4HCO_3 as solvent. Sephadex G-75 and G-100 columns (1.5

x 190 cm) were often used to separate CNBr fragments.

4. Electrophoresis

a. POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn(1969) on slab gels made from 7.5% polyacrylamide containing 0.1% SDS and 6M urea in 100 mM sodium phosphate buffer,pH7.0. Laemmli-urea gel electrophoresis was performed by the method of Laemmli(1970) except that the gel was formed from 15% polyacrylamide, 0.1% SDS and 6M urea in 0.375M Tris-HCl buffer, pH8.8. SDS-Urea 12.5% polyacrylamide gel electophoresis was carried out as described by Swank and Mankres(1971).

Benign gel electrophoresis was carried out as described by Head and Perry(1974), except the gels were polymerized from 7.5% acrylamide in Tris-Glycine buffer, pH8.6, containing 6M urea.

b. HIGH VOLTAGE PAPER ELECTROPHORESIS

The purification of peptide fragments was achieved by a combination of high voltage paper electrophoresis on Whatman # 1 MM filter paper at pH6.5 (pyridine/acetic acid/water, 100:3:900, by vol.) and pH1.8 (formic acid/acetic acid/ water, 1:4:45, by vol.)at 3000 volts for the desired interval. Sometimes an additional pH3.5 electrophoresis (pyridine/acetic acid/water, 1:10:189, by vol.) at 3000 volts for 45 min was required as a final purification. The peptides were detected with the cadmium/ninhydrin reagent (85 ml of 1% ninhyrin in acetone + 15 ml of 1% cadmium acetate in acetic acid).

5. Other Methods

a. S-CARBOXAMIDOMETHYLATION OF PROTEIN SH GROUPS

The general procedure for S-carboxamidomethylation of protein sulfhydryl(SH) groups was performed as follows: all buffer solutions were degassed for 2 hr and then saturated with nitrogen. All reactions were done in the dark. A solution of freshly prepared 20 mM iodoacetamide in denaturing or benign media was added to the protein solution(4 mg/ml) to yield a 4 mM final concentration of iodoacetamide. The reaction mixture was stirred slowly for 30 min, then solid DTT was added to yield a final concentration of 10 mM to quench the excess iodoacetamide. After 30 min, the reaction mixture was desalted on a G-25 Sephadex column (1.6x100 cm) equilibrated with either 1mM HCl or 50 mM NH_4HCO_3. The protein fraction was collected and lyophilized. When radioactive iodoacetamide was used, the $[1-^{14}C]$-iodoacetamide was diluted with iodoacetamide to give a specific activity of 1000 cpm per nmole.

The reaction can be represented as the following equation:

$$\text{Protein-SH} + \text{I-CH}_2\text{-CONH}_2 \text{----> Protein-S-CH}_2\text{CONH}_2 + \text{I}^-$$

b. VISCOSITY MEASUREMENTS

Viscosity measurements of α-TM, CM-Tn, native troponin, and the noncovalent complex of tropomyosin and CM-Tn in 10 mM Tris-HCl, 0.1M KCl buffer, pH7.5, were performed using a Cannon-Manning Semi-Micro Viscometer, type A50, with a flow through time for water of about 5 min. Solution mixtures of tropomyosin and CM-Tn at various molar ratios were allowed to stand for 1 hr at 20°C before the measurements were taken.

c. CYANOGEN BROMIDE CLEAVAGE

Cyanogen bromide cleavage was carried out at room temperature in 70% formic acid using 200 fold molar excess of cyanogen bromide over the number of methionines in in the proteins. After 24 hr the reaction mixture was diluted 10 fold with water and lyophilized to remove excess reagents.

d. AUTORADIOGRAPHY

The ^{14}C-labelled peptides were located on electrophorograms by autoradiography using Kodak X-Omat RP film. The quantities of radioactivity were determined by liquid-scintillation counting of the acid hydrolysates of the pure peptides. The relative intensities of the spots on the autoradiograms were determined by scanning densitometry of the autoradiograms and calculation of the areas under the curves.

e. PHOTOLYSIS

All photolyses were done in a cold room at 4°C in a RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middletown, Conn.) equipped with RPR 3500 A lamps. The photochemical reaction vessel from Ace Glass Incorporated, New Jersey, was maintained at 4°C by a refrigeration system circulating a 50% ethanol-water solution. Also the air surrounding the photolysis apparatus was circulated by an electric fan. The protein solution was transferred into the photochemical reaction vessel and saturated with nitrogen for 1 hr before the photolysis. The protein solution (2-3 mg/ml) was photolyzed for the desired interval and then lyophilized.

III. TOPOGRAPHICAL RELATIONSHIP OF SH GROUPS OF THE TROPONIN COMPLEX

A. Introductory Remarks

The studies in this chapter were undertaken to further characterize the accessibility of the sulfhydryl(SH) groups of rabbit skeletal troponin in the absence and presence of calcium. The role of SH groups of troponin in the calcium control of actomyosin contractivity were first reported by two different research groups(Yasui et al.,1968; Arai and Watanabe,1968). Although the results of both groups were conflicting with regard to the number of SH groups present in the troponin molecule, they agreed that the ability of troponin to interact with tropomyosin in the sensitization of actomyosin was lost if the troponin's SH groups were not protected from oxidation during the protein preparation. These results suggested that non-physiological disulfide formation resulted in a biologically inactive troponin complex which was unable to undergo the correct conformational changes induced by calcium ions.

Today, all three subunits of rabbit skeletal troponin have been sequenced. The primary structures of these subunits have shown that TnT has no cysteine residue(Pearlstone et al.,1976), TnI has three (Wilkinson and Grand, 1978), TnC has one (Collins et al.,1973) for a total of four cysteine residues per troponin complex. Perry(1979) showed that S-carboxamidomethylated TnC and the peptide CB9 (a CNBr fragment of rabbit skeletal TnC, residues 84-135) both retained the following biological activities: an ability to bind calcium with high affinity, to form a complex with TnI that is stable to high urea concentrations and is calcium dependent, to neutralize the inhibition of Mg^{2+}-stimulated actomyosin ATPase produced by TnI, and to inhibit phosphorylation of rabbit skeletal TnI by cyclic AMP-dependent protein kinase. Also, Head and Perry(1974) showed that [^{14}C]carboxamidomethylated TnC could be displaced by unmodified TnC from TnI, suggesting a higher affinity of the untreated TnC for TnI.

Troponin reconstituted with oxidized TnI produced very little calcium sensitivity in the actomyosin ATPase, but this activity could be restored when dithiothreitol was added to the system (Horwitz et al.,1979). Similarly, oxidized TnI will not bind to TnT whereas the reduced TnI will(Horwitz et al.,1979; Hincke et al., 1979).

There is little known about the chemical reactivity of SH groups in native troponin. Potter et al.(1976) showed that there were differences in the reactivity of cysteine 98 of TnC with dansyl fluorescence probe in the presence and absence of Ca^{2+} (84% reduction in the reactivity of cysteine 98 in the presence of Ca^{2+}). They (Potter et al.,1976) also found that the reactivity of cysteine 98 of TnC in the TnI-TnC complex in benign medium with or without Ca^{2+} was significantly reduced compared to that in 6M urea. These results suggest that cysteine 98 of TnC is at the site of interaction with TnI in both the presence and absence of Ca^{2+}, and it may be partially buried in TnC in the presence of Ca^{2+}. Furthermore, Ohyashiki and Sekine (1979) using SH-directed fluorogenic reagents, found that the amount of reagent incorporated in native Tn was the same in the presence and absence of Ca^{2+} (approximately 1 mole reagent per mole of Tn). However, they did not know whether the label was specifically attached to one SH group or to which protein (TnC or TnI).

In this chapter I describe an investigation of the SH groups of TnI for their involvement in the sites of interaction with TnC and or TnT in the presence and absence of calcium and which SH groups can be selectively modified with cross-linking reagents or other structural probes. This information was obtained by the differential labelling with iodoacetamide and [$1^{14}C$]-iodoacetamide in the following skeletal muscle protein complexes: native troponin, TnI-TnC complex, TnI-TnT complex, reconstituted troponin (carboxamidomethylated TnI-TnT complex and TnC), and isolated CM-TnI from these complexes in benign media in the presence and absence of calcium.

B. Experimental Procedures

1. S-carboxamidomethylation of Native Troponin in the Presence or Absence of Calcium

Rabbit skeletal troponin (50 mg) was dissolved in 10 ml of 50 mM Tris-HCl, 0.1M KCl, 1 mM DTT, 3 mM $CaCl_2$ buffer, pH7.5, and stirred for 15 min. The protein solution was dialyzed overnight at 4°C against 1 litre of the same buffer. The S-carboxamido methylation was carried out as described in general methods. In the absence of calcium, 1 mM EGTA was used instead of 3 mM Ca^{2+}.

2. S-carboxamidomethylation of Reconstituted TnI-TnC Binary Complex in the presence or absence of Calcium

A TnI solution (1.4 umol or 30 mg in 5 ml of 1 mM HCl, 0.1M KCl) was added to a solution of TnC (0.7 umol or 13 mg in 3 ml of 100 mM Tris-HCl, 0.1M KCl, 3 mM $CaCl_2$, 1 mM DTT buffer, pH7.5). The solution was stirred for 15 min, and then dialyzed for 8 hr at 4°C against 1 litre of 10 mM K_2HPO_4, 1M KCl, 2 mM β-mercaptoethanol buffer, pH7.0. The protein solution was clarified by centrifugation (20,000 rpm for 10 min). The supernant was loaded onto a 1.0 x 40 cm hydroxylapatite column which was equilibrated with starting buffer 10 mM K_2HPO_4, 1M KCl, 2 mM β-mercaptoethanol buffer, pH7.0. The column was washed with two column volumes of starting buffer at a flow rate of 10 ml per hr. The TnI-TnC complex was eluted with a 60 mM K_2HPO_4, 1M KCl, 2 mM β-mercaptoethanol buffer , pH7.0. The TnI-TnC complex was collected (8 ml) and dialyzed for 8 hr at 4°C against 1 litre of 50 mM Tris-HCl, 0.1M KCl, 1 mM DTT buffer, pH7.5 containing either 3 mM $CaCl_2$ or 1 mM EGTA. The S-carboxamidomethylation was carried out using the general procedure described in Chapter II.

3. S-carboxamidomethylation of Reconstituted TnI-TnT Binary Complex

A 1:1 molar ratio of TnI-TnT complex (15 mg TnT + 10 mg TnI) was dissolved in 5 ml of 50 mM Tris-HCl, 6M urea, 1 mM EGTA, 1 mM DTT buffer, pH7.8. The protein solution was dialyzed at 4°C against 1 litre of 50 mM Tris-HCl, 0.5M KCl, 1 mM DTT buffer, pH7.5 for 8 hr, followed by 1 litre of 50 mM Tris-HCl, 0.3M KCl, 1 mM DTT buffer, pH7.5. The general procedure of S-carboxamidomethylation was performed.

4. S-carboxamidomethylation of Reconstituted Troponin in the Presence of Calcium

A solution containing the S-carboxamidomethylated complex of TnI and TnT (26 mg in 4 ml of 1 mM HCl, 0.2M KCl) was added into a solution containing TnC (10 mg in 3 ml of 100 mM Tris-HCl, 0.2M KCl, 3 mM $CaCl_2$, 1 mM DTT buffer, pH7.5). The solution was stirred for 15 min and then dialyzed overnight at 4°C against 1 litre of 50 mM Tris-HCl, 0.1M KCl, 3 mM $CaCl_2$, 1 mM DTT buffer , pH7.5. The general procedure of S-carboxamidomethylation was carried out.

5. Stepwise S-carboxamidomethylation of Native Troponin in EGTA and Calcium

Native troponin was S-carboxamidomethylated first in an EGTA medium as described above. The S-carboxamidomethylated Tn (20 mg) was dissolved in 5 ml of 50 mM Tris-HCl, 0.1M KCl, 3 mM $CaCl_2$, 1 mM DTT buffer, pH7.5 and dialyzed overnight at 4°C against 1 litre of the same buffer. The general procedure of S-carboxamidomethylation was continued as described previously.

6. S-carboxamidomethylation of Troponin Subunits

Proteins (5 mg) such as TnI, TnC or Cm-TnI were dissolved into 2 ml of 50 mM Tris-HCl, 6M urea, 1mM EGTA, 1 mM DTT buffer, pH7.8. The protein was dialyzed overnight at 4°C against 500 ml of the same buffer and S-carboxamidomethylated using the general procedure.

7. Enzymic Digestions

Tryptic fragments were obtained using a 1:50 molar ratio trypsin-TPCK to protein at 37°C in 50 mM NH_4HCO_3. After 16 hr the reaction mixture was lyophilized.

8. Other Methods

Paper electrophoresis, autoradiography, cyanogen bromide cleavage, amino acid analysis and SDS-urea-polyacrylamide gel electrophoresis were carried out as described in Chapter II.

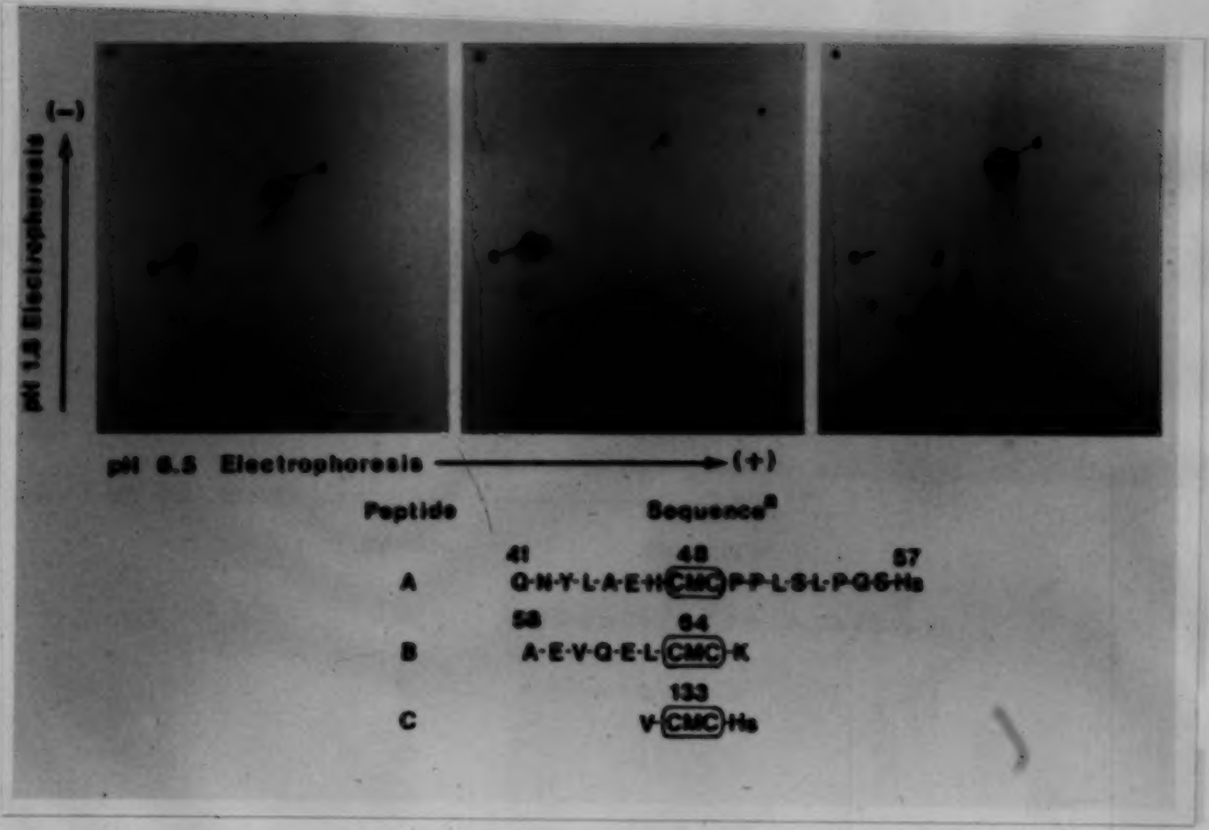

pH 1.8 Electrophoresis
(−)
pH 6.5 Electrophoresis
(+)
Peptide
Sequence^a
A
41
48
57
Q-N-Y-L-A-E-H-CMC-P-P-L-S-L-P-G-S-Hs
B
58
64
A-E-V-Q-E-L-CMC-K
C
133
V-CMC-Hs

Fig. 3.1 Electrophoretic mobilities of radioactive peptides obtained by two-dimensional High Voltage Paper Electrophoresis at pH 6.5 and pH 1.8 of the combined CNBr cleavage and tryptic digestion of ^{14}C-S-carboxamidomethylated troponin I. (a) Troponin I was reacted [1-^{14}C]-iodoacetamide;(b) Native troponin was reacted [1-14C]-iodoacetamide in the presence of calcium; (c) Native troponin was reacted with iodoacetamide in the presence of calcium, followed by isolation of CM-TnI and reaction with [1-14C]-iodoacetamide.

C. Results

1. Isolation of ^{14}C-labelled Peptides from ^{14}C-S-carboxamidomethylated TnI

The amino acid sequence of rabbit skeletal muscle TnI is known (Wilkinson and Grand, 1978). The sequences of the three ^{14}C-S carboxamidomethylated peptides isolated by high voltage paper electrophoresis after a combined CNBr cleavage and tryptic digest of ^{14}C-CM-TnI are shown in Fig. 3.1. The three peptides were well resolved after high voltage electrophoresis at pH6.5 for 1 hr and pH1.8 for 90 min (Fig. 3.1a). The three sulfhydryls were completely accessible to reaction with iodoacetamide whether the S-carboxamidomethylation was carried out in benign medium (50 mM Tris-HCl, 0.3M KCl, 1 mM DTT buffer, pH7.5) or in denaturants (6M urea). Amino acid analysis and radioactivity measurements indicated 3 mole CMC/mole of TnI (Table 3-I). The observed amino acid compositions of the three peptides eluted from the paper are shown in Table 3-II. An additional pH3.5 electrophoresis was required to purify peptide C.

2. Identification of the SH groups Accessible to S-carboxamidomethylation in the following Protein Complexes

a. NATIVE TROPONIN IN THE PRESENCE OF CALCIUM

The general procedure used for the preparation of troponin always resulted in high molecular weight impurities that co-precipitated with troponin in the

40-55% $(NH_4)_2SO_4$ fraction. The troponin was further purified by the method of Reisler et al.(1980) using an Affi-Blue column. Since the troponin had not been treated with any denaturant we can assume the troponin is in the native conformation. The purity of the preparation was verified by SDS gel electrophoresis.

Native troponin consists of a complex of 1 mole of each of the three subunits (TnC, TnI and TnT). When native rabbit skeletal troponin was S-carboxamidomethylated in the presence of calcium with either [1-^{14}C]-iodoacetamide or iodoacetamide, amino acid analyses and radioactivity measurements showed the presence of 1 mole of S-carboxamidomethylated cysteine (CMC) per mole of troponin (Table 3-I). To determine which subunit of the troponin complex was S-carboxamidomethylated, the CM-Tn was separated into its individual components using DEAE-Sephadex A-50 chromatography in the presence of 8M urea and 1 mM EGTA. Radioactive counting indicated that only TnI was ^{14}C-labelled (Fig. 3.2). Thus cysteine 98 of TnC was inaccessible to S-carboxamidomethylation. Amino acid analysis and radioactivity measurements showed 1 mole of CMC per mole of TnI. To identify which cysteine residue of TnI in the troponin complex was exposed, the procedure shown in the Fig. 3.3 was carried out. When ^{14}C-CM-TnI isolated from ^{14}C-CM-Tn was cleaved with CNBr and digested with trypsin , only one ^{14}C-labelled

TABLE 3-I

S-carboxamidomethyl Cysteine (CMC) Content of TnI and TnC in Protein Complexes[a]

Protein or Protein Complexes	TnI Benign Media $-Ca^{2+}$	TnI Benign Media $+Ca^{2+}$	Urea 6M	TnC Benign Media $-Ca^{2+}$	TnC Benign Media $+Ca^{2+}$
TnI	3.0	N.D.	3.0		
Native Tn 1	0.98	1.11		0	0
2[b]	1.00	0.98		0	0
TnI-TnC	3.04	2.20[c]		0	0.05
TnI-TnT	1.16	N.D.			
Reconstituted Tn[d]	N.D.	1.02		N.D.	0

[a] CMC content was determined by amino acid analysis and radioactivity measurements for $[1-^{14}C]$-CMC. The results represent moles CMC per mole of TnI or TnC.

[b] Native Tn was stepwise S-carboxamidomethylated with iodoacetamide in EGTA and then the carboxamidomethylation repeated with $[1-^{14}C]$-iodoacetamide in calcium.

[c] This value was obtained from three separate experiments. If the isolated CM-TnI from the CM-TnI-TnC complex was recarboxamidomethylated, a value of 3.0 mole of CMC per mole of TnI was obtained.

[d] Tn was reconstituted from nonradioactive carboxamidomethylated TnI-TnT complex with TnC in 1:1:1 molar ratio of TnI:TnT:TnC. This reconstituted complex was recarboxamidomethylated with $[1-^{14}C]$-iodoacetamide. No incorporation of radioactivity and no further increase in CMC content was observed over that found for the TnI-TnT complex.

peptide was identified by high voltage paper electrophoresis, autoradiography, and amino acid analysis (peptide C, Fig. 3.1b). This result suggested that in the presence of Ca^{2+},cysteine 133 of TnI in the native troponin was exposed. To verify this result non-radioactive CM-TnI isolated from CM-Tn was reacted with [1-^{14}C]-iodoacetamide in 6M urea. The ^{14}C-labelled peptides were isolated and identified as described in Fig. 3.3 as peptides A and B (Fig. 3.1c). This result shows that cysteines 48 and 64 of TnI in native troponin were inaccessible to S-carboxamidomethylation in the presence of calcium, either protected by the TnT and/or TnC interactions or buried as a result of conformational changes induced by TnT and/or TnC.

b. NATIVE TROPONIN IN THE PRESENCE OF EGTA

When native troponin was S-carboxamidomethylated in the presence of 1 mM EGTA as described in Fig. 3.3, the following results were obtained:Firstly, when [1-^{14}C]-iodoacetamide was reacted with native troponin , only 1 mole of CMC per mole of troponin was found (Table 3-I). After separation of ^{14}C-CM-Tn,into its components on DEAE-Sephadex chromatography ,only TnI was ^{14}C-labelled. Thus cysteine 98 of TnC was inaccessible to carbox- amidomethylation. The ^{14}C-labelled peptide was identified as peptide C (Fig. 3.1b) indicating that cysteine 133 of TnI was exposed in native troponin in

TABLE 3-II

Amino Acid Compositions of the
^{14}C-S-carboxamidomethylated Peptides of TnI[a]

Amino Acid	A	Peptides[b] B	C
CMC[c]	0.99 (1)	0.96 (1)	1.00 (1)
Asx	1.03 (1)		
Ser	1.92 (2)		
Hse	0.99 (1)		0.98 (1)
Glx	2.00 (2)	3.04 (3)	
Pro	3.14 (3)		
Gly	1.15 (1)		
Ala	1.03 (1)	0.82 (1)	
Val		1.23 (1)	1.01 (1)
Leu	2.83 (3)	0.88 (1)	
Tyr	0.90 (1)		
Lys		1.06 (1)	
His	1.03 (1)		

[a] Residues per mole: Integral values in parentheses were obtained from the sequence of rabbit skeletal TnI (Wilkinson and Grand, 1978).

[b] ^{14}C-CM-TnI was cleaved with CNBr followed by tryptic digestion. The peptides were purified by paper electrophoresis at pH 1.8, 3.5, 6.5.

[c] CMC content was determined by radioactivity measurements and amino acid analysis.

the absence of calcium. Secondly, when native Tn was first reacted with iodoacetamide, followed by isolation of CM-TnI which was then reacted with [1-^{14}C]-iodoacetamide in 6M urea two ^{14}C-labelled peptides were isolated and identified as peptides A and B (Fig. 3.1c). This result confirms the exposure of cysteine 133 of TnI in native Tn in the absence of calcium and cysteines 48 and 64 of TnI along with cysteine 98 of TnC are inaccessible to the reaction with iodoacetamide.

c. RECONSTITUTED TnI-TnC COMPLEX IN THE PRESENCE OF CALCIUM

To determine the SH groups accessible to S-carboxamidomethylation in the TnI-TnC complex in the presence of Ca^{2+}, the experiment was carried out using the following three procedures: formation of the TnI-TnC complex by mixing a 1:1 molar ratio of TnI and TnC in a 50 mM Tris-HCl, 0.1M KCl, 1 mM DTT buffer, pH7.5; isolation of the complex from DEAE-chromatography in the presence of calcium; isolation of the complex from hydroxylapatite chromatography as described in the "Experimental Procedures" (Fig. 3.4). No difference in the results were observed. When the TnI-TnC complex was reacted in the presence of calcium with [1-^{14}C]iodoacetamide and then separated into its individual components using DEAE-Sephadex

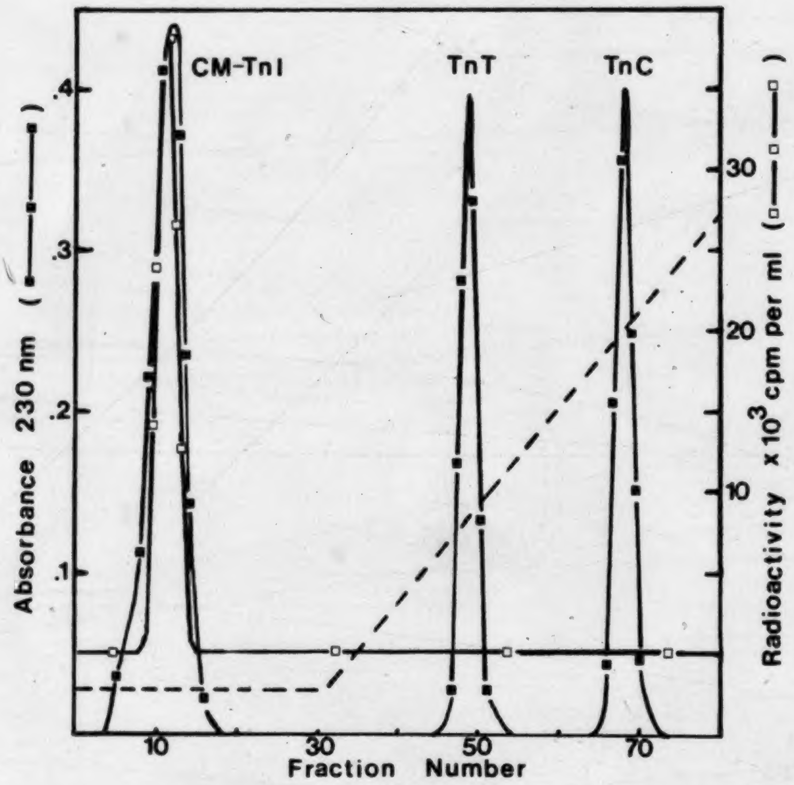


Fig. 3.2. Chromatographic Separation on DEAE A-50 Sephadex of Native Troponin which had been ^{14}C-S-carboxamidomethylated in the presence of calcium. The column (1.6 x 35 cm) equilibrated with 50 mM Tris-HCl, 6M urea, 1 mM EGTA, 1 mM DTT buffer, pH 7.8, was eluted with a linear salt gradient (- - -) from 0 to 0.6M KCl in the same buffer (150 ml each). The column effluent was monitored for absorbance at 230 nm (■-■-■-■) and radioactivity (□-□-□-□).

chromatography, radioactivity was observed mainly in TnI (Fig. 3.5). Amino acid analysis of TnC showed less than 0.05 mole CMC per mole of TnC (Table 3-I). This result suggests that cysteine 98 of TnC was inaccessible to S-carboxamidomethylation and either buried in TnC by conformational changes in TnC induced by TnI or protected by the interaction with TnI.

The ^{14}C-CM-TnI isolated from the TnI-TnC complex was fragmented and the radio-labelled peptides isolated as described in Fig. 3.3. All three cysteine residues of TnI(48, 64 and 133) were accessible to S-carboxamidomethylation, however scanning densitometry of the pH6.5 autoradiogram indicated relative spot intensities of 1.0:0.5:0.6 for cysteines 64, 48 and 133 respectively (Fig. 3.6). This result is in excellent agreement with the amino acid analysis of ^{14}C-CM-TnI which showed 2.2 moles of CMC per mole of TnI (Table 3-I). This result also suggests that TnC, in the presence of calcium may induce some conformational changes in TnI such that cysteines 48 and 133 are less reactive with iodoacetamide.

S-carboxamidomethylation of TnI-TnC complex in the presence of Ca^{2+} with nonradioactive iodoacetamide followed by isolation of CM-TnI and S-carboxamidomethylation of CM-TnI in 6M urea with [1-^{14}C]-iodoacetamide showed an increase in the CMC content from 2.2 to 3 moles of CMC per mole of TnI. The

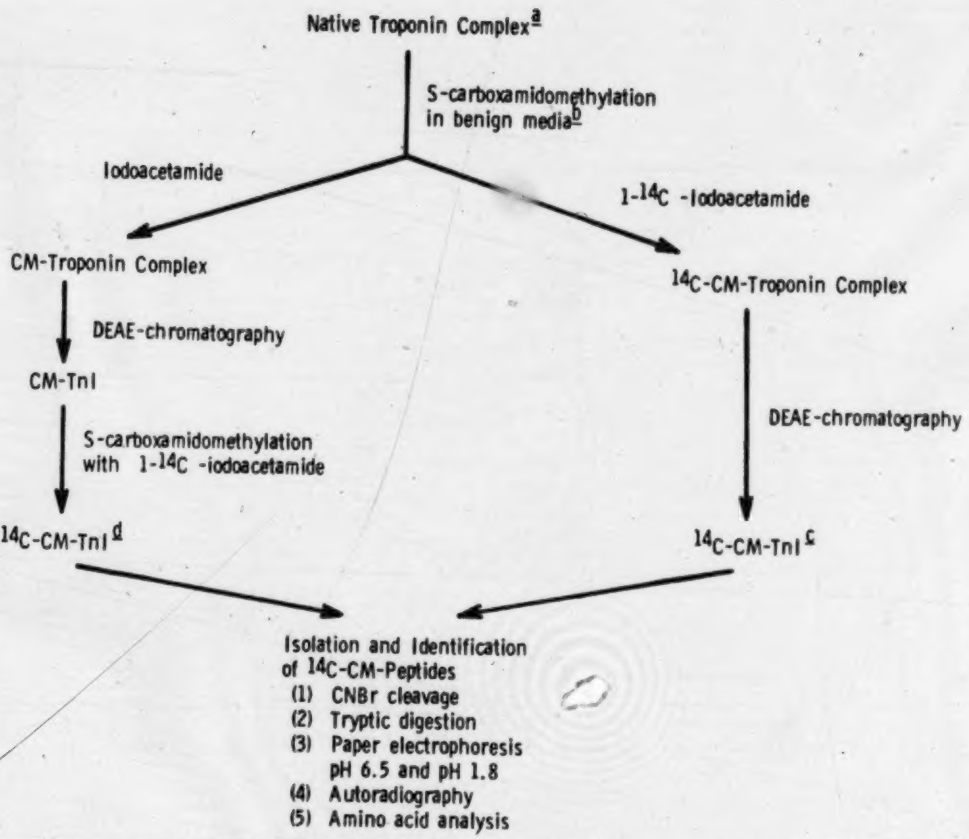

Native Troponin Complex$^{\underline{a}}$
S-carboxamidomethylation in benign media$^{\underline{b}}$
Iodoacetamide
1-^{14}C -Iodoacetamide
CM-Troponin Complex
^{14}C-CM-Troponin Complex
DEAE-chromatography
CM-TnI
S-carboxamidomethylation with 1-^{14}C -iodoacetamide
DEAE-chromatography
^{14}C-CM-TnI$^{\underline{d}}$
^{14}C-CM-TnI$^{\underline{c}}$
Isolation and Identification of ^{14}C-CM-Peptides
(1) CNBr cleavage
(2) Tryptic digestion
(3) Paper electrophoresis pH 6.5 and pH 1.8
(4) Autoradiography
(5) Amino acid analysis

Fig. 3.3. Scheme for the identification of the SH groups of native troponin acessible to S-carboxamidomethylation. (a), isolation of native troponin, S-carboxamidomethylation, DEAE-chromatography, CNBr cleavage, tryptic digestion, paper electrophoresis and autoradiography are described under "Experimental Procedures"; (b), 50 mM Tris-HCl, 0.1M KCL, 1 mM DTT buffer, pH 7.5 in the presence of either 1 mM EGTA or 3 mM $CaCl_2$; (c), only the SH groups of TnI in native troponin accessibe to S-carboxamidomethylation are ^{14}C-labelled; (d), SH groups of TnI inaccessible in native troponin to S-carboamidomethylation are ^{14}C-labelled.

autoradiogram also showed only two weak radioactive spots corresponding to peptides containing cysteines 48 and 133.

d. RECONSTITUTED TnI-TnC COMPLEX IN THE ABSENCE OF CALCIUM

The formation of a TnI-TnC complex in the presence of EGTA has been demonstrated previously by many workers (Greaser and Gergly, 1973; VanErd and Kawasaki,1973; Potter and Gergley,1974; McCubbin et al.,1974; Hitchcock,1975; and Sutoh,1980). The formation of the complex was demonstrated here by the solubilization of TnI by TnC when mixing the TnC and TnI solutions in 0.5M KCl in 1:1 molar ratio. The TnI remained soluble on reducing the KCl concentration by dialysis to 0.1M. Further support for this interaction in EGTA comes from the fact that S-carboxamidomethylation of the TnI-TnC complex with [1-^{14}C]-iodoacetamide and separation of the individual components on DEAE-Sephadex chromatography showed no radioactivity in the TnC fraction and no CMC content in the TnC by amino acid analysis. This result suggests that TnI renders cysteine 98 of TnC inaccessible to reaction with iodoacetamide since under the same conditions without TnI, cysteine 98 of TnC can be completely labelled with the photoaffinity probe AGTC (see Chapter V) and other structural probes (Potter et al.,1976). Amino acid analysis of isolated CM-TnI from

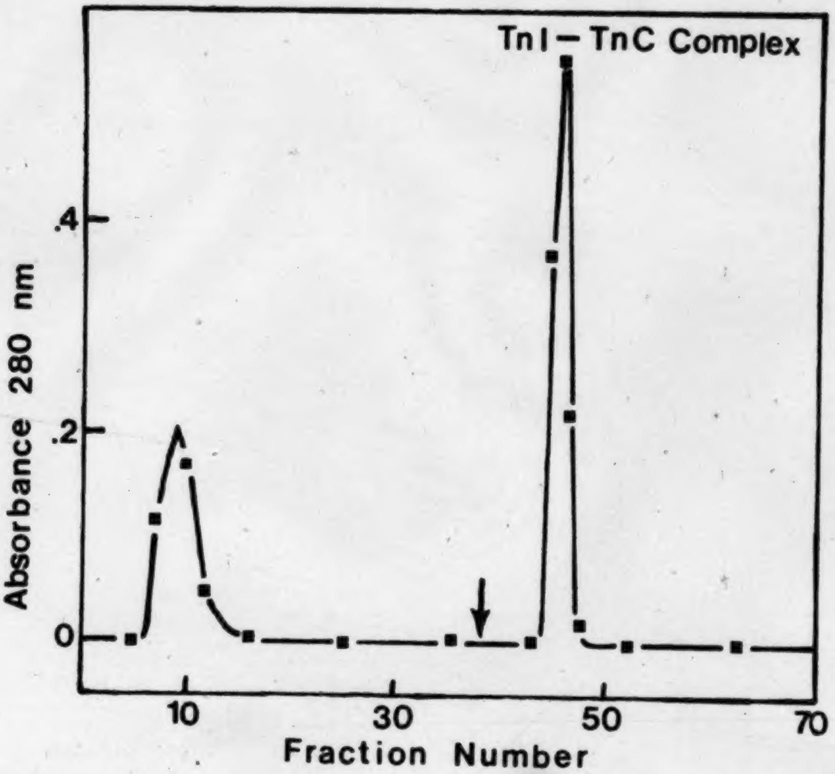


Fig. 3.4. Chromatographic isolation of TnI-TnC binary complex on a hydroxylapatite column. The conditions of chromatography have been described in the "Experimental Procedures". The first peak eluted off the column with the 10 mM K_2HPO_4, 2 mM β-mercaptoethanol, 1M KCl, pH7.0 buffer was TnI(excess TnI was used to ensure that all TnC was complexed with TnI). The arrow indicated the start of the 60 mM K_2HPO_4, 2 mM β-mercaptoethanol, 1M KCl, pH7.0 buffer used to elute the TnI-TnC complex. The 1:1 ratio of TnI to TnC in the complex was verified by amino acid analysis and SDS-gel electrophoresis.

^{14}C-S-carboxamidomethylated TnI-TnC complex in the presence of EGTA showed 3 moles of CMC per mole of TnI (Table 3-I). The ^{14}C-CM-TnI was fragmented and the radiolabelled peptides was isolated as described in Fig. 3.3. The autoradiogram showed 3 radioactive spots corresponding to the three cysteine residues of TnI. The relative intensities of the spots in the pH6.5 autoradiogram were 1:1:1. S-carboxamidomethlyation of the TnI-TnC complex in EGTA, followed by isolation of CM-TnI and S-carboxamidomethylation of CM-TnI with [1-^{14}C]iodoacetamide showed no radioactivity in TnI. These results suggested that all three cysteines were exposed in the TnI-TnC complex in the presence of EGTA. The difference in results, that is cysteines 48 and 133 less reactive in the presence of Ca^{2+} to completely reactive in the absence of Ca^{2+} can be explained by conformational changes in the TnI-TnC complex induced by Ca^{2+}.

e. RECONSTITUTED TnI-TnT COMPLEX

Interactions between TnI and TnT have been demonstrated (Horwitz et al.,1979; Hincke et al.,1979; Katayama,1979; Pearlstone and Smillie,1980). To determine the SH groups accessible to S-carboxamidomethylation in the TnI-TnT complex (1:1 molar ratio), the procedure outlined in Fig 3.3 was used. Amino acid analysis of the isolated CM-TnI from

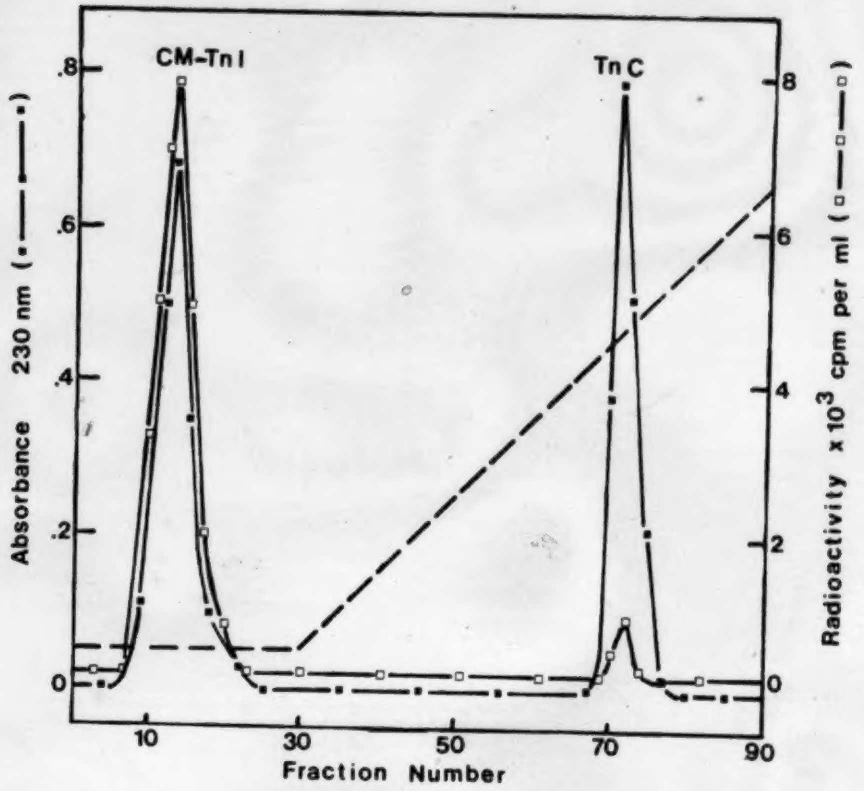


Fig. 3.5. Chromatographic separation on DEAE A-50 Sephadex of TnI-TnC complex which had been ^{14}C-S-carboxamidomethylated in the presence of calcium. The column (1.6 x 35 cm) equilibrated with 50 mM Tris-HCl, 6M urea, 1 mM EGTA, 1 mM DTT buffer, pH7.8 was eluted with a linear salt gradient (- - -) from 0 to 0.6M KCl in the same buffer (150 ml each). The column effluent was monitored for absorbance at 230 nm (■-■-■) and radioactivity (□-□-□).

the ^{14}C-S-carboxamidomethylated TnI-TnT complex showed 1.16 mole of CMC per mole of TnI (Table 3-I). S-carboxamidomethylation with [1-^{14}C]-iodoacetamide of the TnI-TnT complex followed by isolation and identification of the ^{14}C-labelled peptides of ^{14}C-CM-TnI indicated that cysteine 133 was modified. Trace amounts of radioactivity were observed in peptides containing cysteine 48 and 64. This trace labelling can be explained by the presence of some uncomplexed TnI in the mixture. S-carboxamidomethylation of the TnI-TnT complex followed by isolation of CM-TnI and S-carboxamidomethylation of CM-TnI with [1-^{14}C]-iodoacetamide showed 3 moles of CMC per mole of TnI. After separation of the ^{14}C- labelled peptides two radioactive spots corresponding to cysteines 48 and 64 of TnI were identified on the autoradiogram. These results suggest that cysteine 133 is exposed in the TnI-TnT complex while cysteine residues 48 and 64 of TnI were either protected by TnT upon interaction or that TnT induced a conformational change in TnI on binding such that the cysteine residues 48 and 64 are buried in the TnI molecule and unavailable for reaction with iodoacetamide.

f. RECONSTITUTED TROPONIN IN THE PRESENCE OF CALCIUM

To verify the results obtained in both native troponin and in the TnI-TnT complex, we first

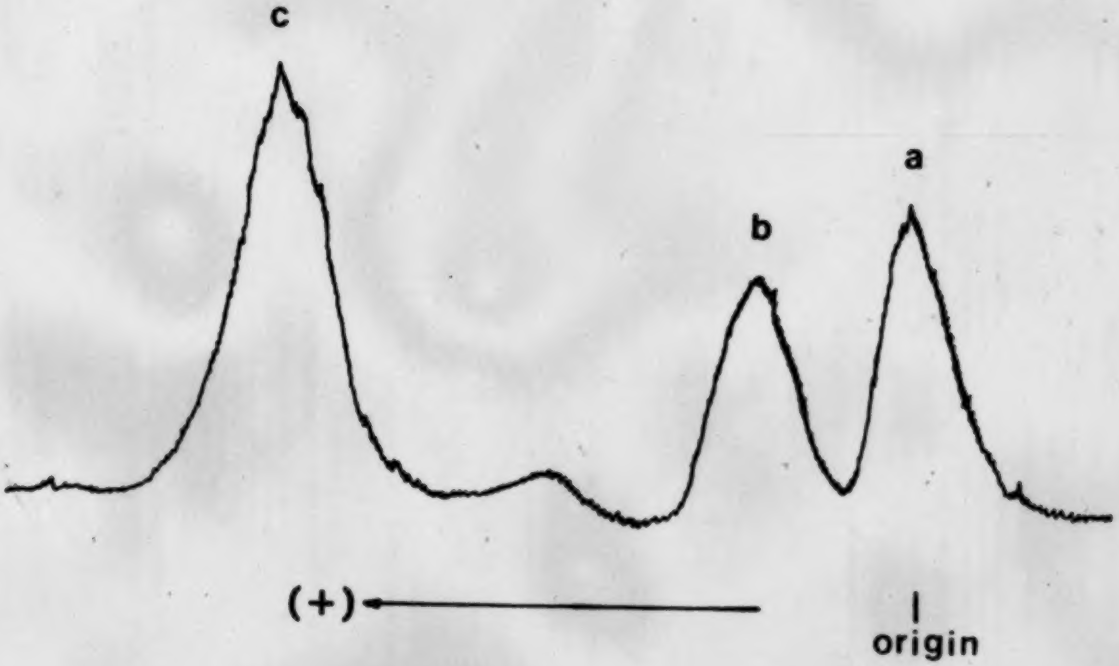


Fig. 3.6 Densitometric traces of the pH 6.5 autoradiogram of ^{14}C-S-carboxamidomethylated peptides from ^{14}C-labelled CM-TnI. (a) CM-cysteine 133 radiolabelled peptide; (b) CM-cysteine 48 radiolabelled peptide; (c) CM-cysteine 64 radiolabelled peptide. The relative spot intensities are 0.58; 0.5; 1.0 for (a); (b); and (c) respectively.

S-carboxamidomethyaled the TnI-TnT complex with non-radioactive iodoacetamide. This complex was reconstituted with TnC in the presence of Ca^{2+} to form the modified troponin complex. This reconstituted troponin was then modified by reacting with $[1-^{14}C]$-iodoacetamide or iodoacetamide. Amino acid analysis showed no increase in the amount of CMC (Table 3-I) and no incorporation of radioactivity.

Verification of the formation of the ternary complex comes from the solubilization of the CM-(TnI-TnT) complex by TnC in the 0.1M KCl and the fact that no CMC or radioactivity was detected in TnC. After S-carboxamidomethylation of the reconstituted troponin complex the CM-TnI was isolated on DEAE-Sephadex chromatography and reacted with ^{14}C-iodoacetamide in 6M urea. Peptide mapping showed two ^{14}C-peptides corresponding to peptides containing cysteine 48 and 64.

D. Discussion

The results of monitoring the accessibility of cysteine residues to react with iodoacetamide in native troponin, various binary and ternary reconstituted complexes in the presence and absence of Ca^{2+} are summarized in Fig 3.7. When cysteine residues are protected from reaction with iodoacetamide by a particular troponin subunit, protection will refer to one of two possibilities: firstly, steric blocking which means the SH groups are at the site of interaction, or, secondly, buried in its own troponin subunit by the binding of another subunit at a site removed from the vicinity of the SH group. Wherever possible we will distinguish between these two possibilities.

Cysteine 98 of rabbit skeletal TnC is protected in native troponin and the TnI-TnC complex in the presence of calcium. In this case cysteine 98 is at the site of interaction. This conclusion is supported by binding studies of proteolytic fragments of TnC where CB9 (residues 84-135) and larger fragments bind to TnI (Leavis et al.,1978; Weeks and Perry,1978) in the presence of calcium. Complex formation with TnI in the presence of Ca^{2+} around cysteine 98 is also indicated by the increase in dansyl fluorescence when cysteine 98 of CB9 is labelled with dansyl chloride (Potter et al.,1976). After attachment of the photoaffinity probe AGTC to cysteine 98 of TnC, a covalently cross-linked binary TnI-TnC complex can be formed upon photolysis (see Chapter V). All proteolytic fragments of TnC which bind to

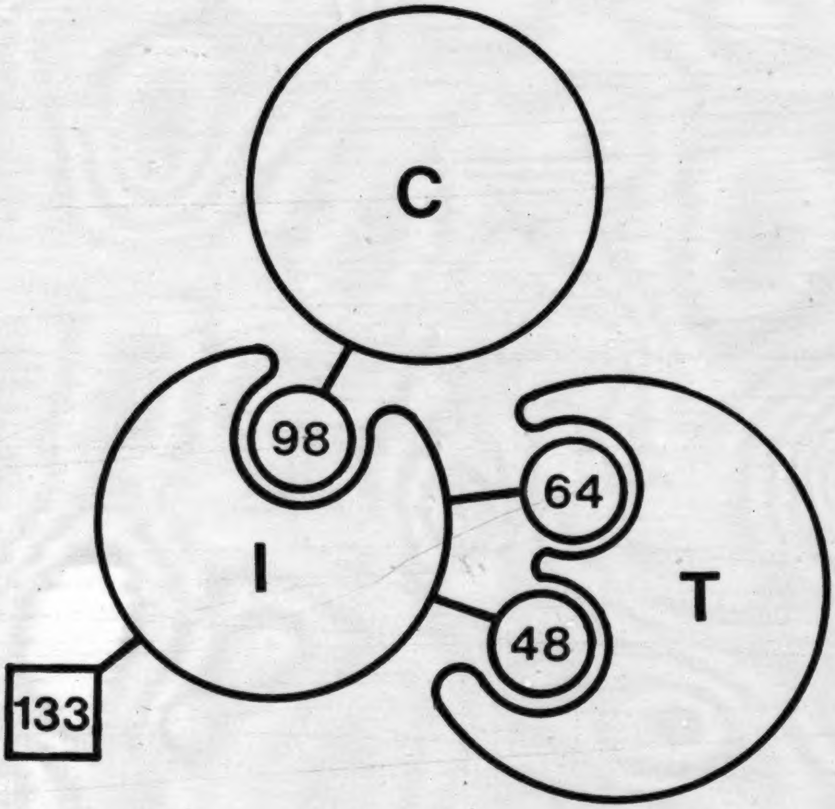


Fig. 3.7. A schematic representation of the troponin complex from rabbit skeletal muscle in the absence and presence of Ca^{2+} showing the exposed (□) and inaccessible (○) SH groups to reaction with iodoacetamide. The symbolism for the proteins is as follows: C, TnC; I, TnI; T, TnT. The numbers represent the positions of the SH groups in the sequences of TnI and TnC. TnT has no SH group.

TnI independent of calcium, all possess a common segment of the amino acid sequence, residues 89-100 (Leavis et al.,1980). It was suggested by Leavis et al.(1978) that residues 89-100 constitute a Ca^{2+}independent link between TnC and TnI holding the complex together. This conclusion is supported by our studies where cysteine 98 is protected in native troponin and the TnI-TnC complex in the presence of EGTA from reacting with iodoacetamide. Similar results were obtained by Potter et al.(1976) where titration of cysteine 98 of TnC in the TnI-TnC complex in the presence and absence of calcium with 5,5-dithiobis(2-nitrobenzoic acid) showed reduction in reactivity compared to titration in 6M urea. Though cysteine 98 of TnC is at the site of interaction with TnI , there are differences in the reactivity of cysteine 98 when TnC alone is modified with sulfhydryl reagents in the absence and presence of calcium in the benign media. Potter and coworker showed an 84% reduction in reactivity of cysteine 98 in the presence of calcium. We have also found that a complete labelling of cysteine 98 of TnC occurs in the presence of EGTA(>95%) with the photoaffinty probe AGTC while only 30% labelling is found in the presence of calcium (see Chapter V). These results would suggest that though cysteine 98 is at the site of interaction with TnI in both the presence and absence of calcium, it may be already partially buried in TnC at the TnI interaction site in the presence of calcium.

Cysteines 48 and 64 of TnI are protected in native troponin in the presence and absence of Ca^{2+} and in the TnI-TnT complex. One can conclude from this result that TnT protects cysteines 48 and 64. Support for the view that the TnT interaction sterically blocks one or both of these cysteines results from the observation that when these SH groups were modified with the photoaffinity probe AGTC a convalently linked TnI-TnT complex was formed upon photolysis (see Chapter VII). These results suggest that TnI is in close contact with TnT in the presence or absence of calcium and are in agreement with previous reports (Horwitz et al.,1979; Hincke et al.,1979; Katayama,1979; Pearlstone and Smillie, 1980). Hitchcock(1981), using a competitive labelling procedure of lysines with acetic anhydride, showed that in the native troponin complex lysines 40, 65, 70, 78 of TnI and in the TnI-TnT complex lysines 40, 65, and 78 of TnI are less reactive. These results are in agreement with our finding that cysteines 48 and 64 of TnI are protected by TnT interaction in the TnI-TnT complex and in native troponin in the presence and absence of Ca^{2+}.

Cysteine 133 of TnI is exposed in native troponin in the presence and absence of calcium and in the TnI-TnT complex. Cysteine 133 is close to the inhibitory region on TnI (residues 105-114) which binds to actin-tropomyosin in the absence of calcium and most likely to TnC in the presence of calcium (Perry, 1979; Talbot and Hodges, 1981).

All four cysteine residues in native troponin are insensitive to calcium induced conformational changes as far as their accessibility to reacting with iodoacetamide. These results agree with two previous studies involving reconstituted troponin by Sutoh(1980) and Hitchcock(1975) using the methyl 4-azidobenzimidate and diimido ester crosslinkers, respectively, where they failed to detect any appreciable calcium dependent change in the topology of troponin free in solution. However Sutoh(1980) did show that the quaternary structure of reconstituted troponin was calcium sensitive in the thin filament complex. Ohyashiki and Sekine(1979) using SH-directed fluorogenic reagents, found that the amount of reagent incorporated into native troponin was the same in the presence and absence of calcium (approximately 1 mole per mole of troponin). However they did not know whether the label was specifically attached to one SH group or to which protein (TnC or TnI). We now know that this sulfhydryl group is cysteine 133 of TnI. Since they did show a significant difference in fluorescence intensity in the presence and absence of calcium which suggests a difference in microenvironment around the label it should be possible to detect such changes using the SH group photoaffinity probes.

The results in this Chapter will allow the selective attachment of sulfhydryl directed reagents for studying the conformational changes in troponin during muscle contraction. Studies involving the selective modification of these SH groups with a crosslinking reagent like AGTC (see Chapter IV) should reveal the spatial orientation of the proteins in the thin filament and thus the molecular mechanism of relaxation and contraction triggered by calcium.

IV. DESIGN, SYNTHESIS AND CHARACTERIZATION OF HETEROBIFUNCTIONAL PHOTOAFFINITY PROBES, AGTC AND ATC

A. Historical Background of Affinity Labelling Technique

Protein structural studies using chemical cross-linking techniques began in the fifties and became widely used as affinity labelling in sixties for identification of amino acid residues involved in the binding and catalytic sites of an enzyme (Wold,1972). The method of affinity labelling is based upon the fact that the binding of most biological ligands to their specific receptor sites involves a number of favorable interactions that together make up the ligand-receptor recognition process. Therefore the total binding free energy is usually high enough to allow a small modification in the structure of the natural ligand without significantly scarifying either the selectivity or the strength of the overall binding. The classical method of affinity labelling takes advantage of this by incorporating a chemically labile functional group into the ligand and cross-linking the modified ligand to the binding site through a chemical reaction. The most used chemically labile functional groups have been discussed by Wold(1972). Although there was some success in using such labile functional groups for labelling the binding or catalytic sites of an enzyme, there are several limitations to this type of approach:

i) Most of the functional groups are unstable in aqueous solutions at pH7-9. For example the imidoester has a half life of 10 min at 4°C.

ii) No control of the chemical reactivity of the labile groups. For example, how would one label the inside components of a cell or a vesicle, without modifying the outside?

iii) In most cases, the efficiency of cross-linking depends upon the appropriate choice of pH and temperature, so it is difficult to accomodate the cross-linking condition with the ligand-receptor binding conditions.

iv) Moreover, successful cross-linking depends on the availability of suitable reactive groups (cysteine, lysine, arginine, histidine, glutamate, aspartate,etc.) located within the effective range of the reagents.

The above problems have hindered development of the site specific affinity labelling. However, in the late sixties several reseach groups suggested that photoactivated functional groups could be used as affinity probes. They also indicated that photoactivated reagents in principle do not have the problems present in the chemical labile functional groups for the following reasons:

i) photo-generated reagents are chemically inert until photolysis which allows control of when cross-linking should take place;

ii) the photoactivated species react indiscriminately at the binding site and are not depended on a specific functional group. This should increase the efficiency of cross-linking.

In other words, one could incorporate the photoaffinity ligand into the binding site, then without changing the binding conditions wash away all excess and unbound ligand. After removal of excess ligand, the control experiments could be performed,such as the measurement of enzymatic activities, the measurement of hormonal stimulation in cellular activities, or the determination of antigen-antibody binding constants. The final step involves irradiation of the sample which converts the photolabile group into a highly chemically reactive species and results in cross-linking within a matter of seconds.

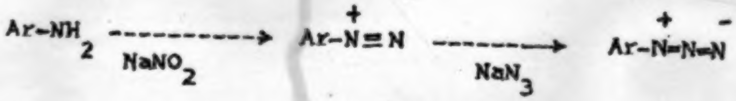
Generally, the most common photoactivated chemical reactive species can be classified into three major functional groups: carbenes from diazo compounds; nitrenes from azido compounds and triplet state diradicals from keto compounds. A good review of the photochemistry and biochemical applications of these three groups has been given by Bayley and Knowles(1977). In this Chapter I will briefly discuss the photochemistry and organic synthesis of azido compounds.

B. Photochemistry of Aryl Azido Reagents

1. Chemical Properties of The Aryl Azide

a. ORGANIC SYNTHESIS

The most convenient method for preparation of an aryl azido compound is diazotization of an aromatic amine followed by treatment with azide ions (Smith, 1970).



$$Ar{-}NH_2 \xrightarrow[NaNO_2]{} Ar{-}\overset{+}{N}{\equiv}N \xrightarrow[NaN_3]{} Ar{-}N{=}\overset{+}{N}{=}\overset{-}{N}$$

The acidic medium can range from 0.1N to 2N sulfuric acid (in the case of sensitive compounds 0.1N sulfuric acid is usually used). Sodium nitrite is used in slight excess and dissolved in cold aqueous solution before it is added to the aromatic amine solution. The diazotization is generally finished within 30 min, and urea is usually used to remove excess nitrite. One equivalent of aqueous sodium azide is added slowly into the mixture, which is stirred at room temperature for one to two hours. The azido compound precipitates and can be recrystallized from appropriate solvents. The yield ranges from 60-80%. Since many biological molecules do not contain aryl amino groups that can be derivatized, some useful reagents for attaching photolabile groups to such molecules have been described by Bayley and Knowles(1977).

b. SPECTROSCOPIC PROPERTIES

The aryl azides have strong ultraviolet absorption often with a peak around 255-278 nm and a characteristic shoulder on the long wavelength side of the peak (285-290 nm). For example, 4-azidobenzoic acid ester has maximium absorbance at 276 nm and 4-azido -N-methyl-2-nitro-aniline has its maxima at 258 nm and 458 nm (Fleet et al., 1972). These bands usually disappear or are considerably reduced on photolysis.

Azido compounds also have a characteristic infrared absorption band at approximately 2100 cm^{-1} (strong asymmetric doublet). The mass spectrum of phenyl azide is reported to have a base peak at m/e 91 corresponding to the loss of a nitrogen molecule. This is followed by the loss of HCN to yield the $C_5H_4^+$ ion (Crow and Wentrup, 1967).

2. Photolysis Conditions

Photolysis should be performed under an atmosphere of nitrogen to prevent chromophore-sensitized photoxidation of the biological system. The solution should also be purged with nitrogen gas before photolysis.

Photolysis at short wavelengths (254 nm) has undesired side effects such as destruction of tryptophan, tyrosine, cysteine and cystine residues (Galardy, 1973). However, these photosensitive residues are stable under long wavelength irradiation (over 300 nm) (Galardy, 1973). The temperature at which photolysis of azides is carried out can affect the cross-linking yield, lower temperature improving the cross-linking probably by increasing the binding constant (Bayley and Knowles, 1977).

3. Reaction Mechanisms

The processes involved in aryl azide photolyses have been studied extensively by Reiser and his coworker(1971). They found that the photoadducts depended upon the photolysis conditions. The fate of aryl azide is summarized in Fig.4.1. Possible and productive reaction mechanisms will be discussed below.

A salient feature of the photochemistry of organic azides is the facile elimination of molecular nitrogen from the azido group on irradiation.

$$R\text{-}N_3 \xrightarrow{h\nu} R\ddot{N}\downarrow\uparrow \quad + N_2$$

The primary product of this process is formally a derivative of monovalent nitrogen and has been termed nitrene.

The actual processes involved in the formation of aryl nitrenes are not clear. However, Reiser and Merley (1968) felt that the best explanation of the photolysis was bond absorption resulting in $\pi \rightarrow \pi^*$ excited state in a vibration level not sufficient to induce bond dissociation. The excited molecule would then undergo rapid internal conversion to lower excited states and finally to a vibrationally excited ground state in which transmission of

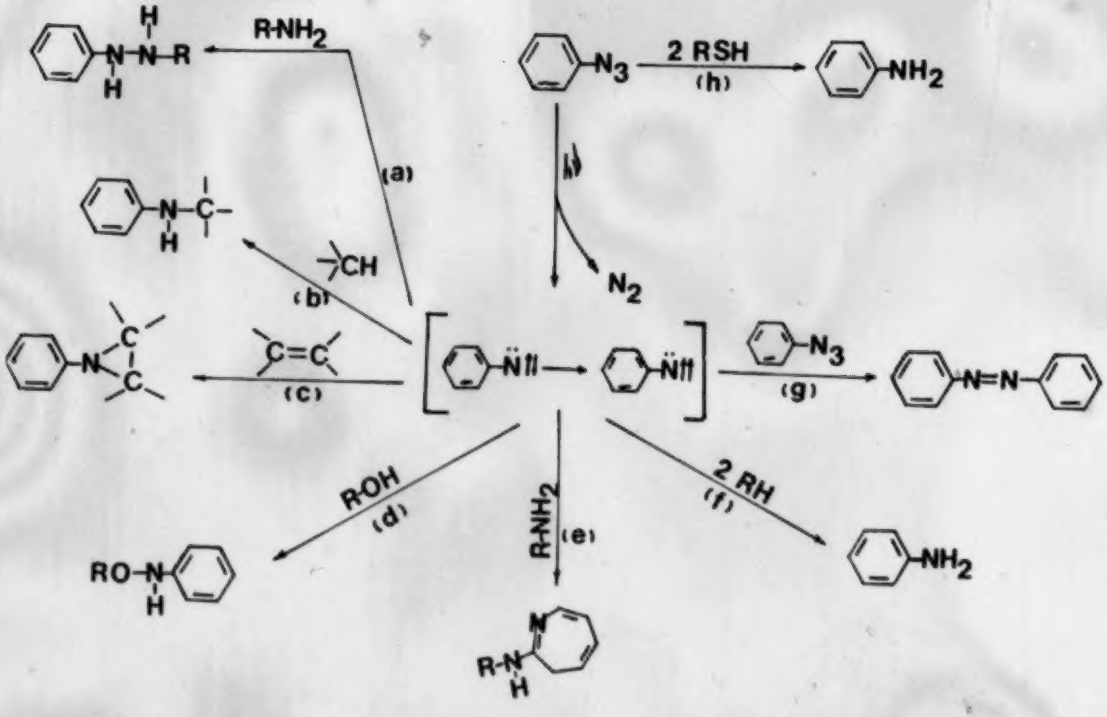

R-NH2
(a)
2 RSH
(h)
N3
NH2
hν
N2
CH
(b)
C=C
(c)
N3
(g)
N=N
R-OH
(d)
R-NH2
(e)
2 RH
(f)
NH2
RO-N-H
R-N-H

Fig. 4.1 Possible reaction mechanisms of Aryl azides. (a), (b), (c) and (d) are covalent adducts of insertion reactions. Non-productive reactions include formation of primary aryl amine (f) and (h). (b) also can occur by a Hydrogen abstraction mechanism.

vibrational momentum and energy from the aromatic skeleton to the azide side chain would lead to dissociation of the N-N bond to give the nitrene.

Of the five valence electrons of the nitrene, one electron takes part in the σ-bond to carbon, one pair occupies the non-bonding sp-orbital and the two remaining electrons are in the unhybridized orbitals Px and Py (Fig. 4.2). The two p-orbitals are equivalent in energy and will carry one electron each, both electrons having the same spin: the ground state of the nitrenes is expected to be a triplet. This hypothesis is supported by Smolinsky et al.(1962) who have observed e.s.r. signals of the triplet state on irradiating the azide at 77°K. However the chemically reactive species of nitrenes can be either the excited triplet or singlet state. It is found that singlet nitrene which is generated from photolysis of aryl azide could produce stereospecific addition products. On the other hand, a triplet nitrene would produce a non-stereo specific addition product (Resier and Wagner, 1970). It is because the triplet reactant can accept only one electron at a time and will close the bond with the second electron only after one of the spins has inverted. The waiting period between the first and the second step is long enough to allow equilibration of the conformers of the transition state (Woodworth and Skell, 1959).

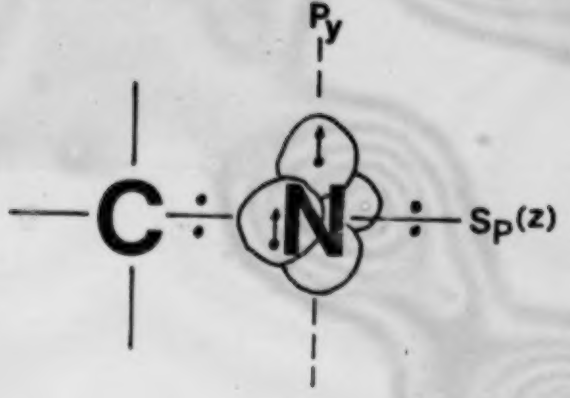
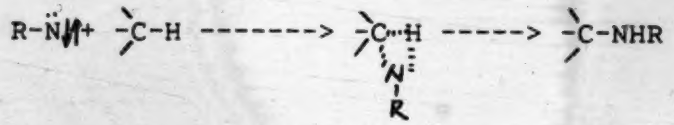


Fig 4.2 Electronic configuration of triplet nitrene. [From Reiser and Wagner, 1971]

a. INSERTION REACTION

Insertion into C-H bonds is exclusively a reaction of singlet nitrenes. Both thermally and photolytically generated nitrenes undergo insertion, the yield of secondary amine depends on both the substrate and the rate of competing processes.



$$R\text{-}\ddot{N}{\uparrow\downarrow} + \text{-C-H} \dashrightarrow \text{-C}\cdots\text{H} \;(\cdots\text{N-R}) \dashrightarrow \text{-C-NHR}$$

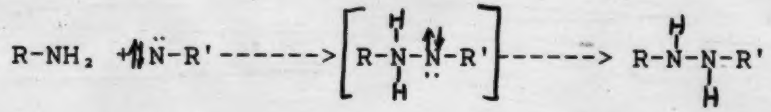
There is preferential insertion into tertiary C-H bonds, followed by secondary and primary C-H bonds: the relative reactivities are 30:10:1(Reiser and Wagner, 1971).

Insertion into O-H bonds with the formation of hydroxyamine derivative was observed(Reiser and Wagner, 1971).

$$R\text{-OH} + R'\text{-}\ddot{N}{\uparrow\downarrow} \dashrightarrow R\text{-O-NHR}'$$

This reaction could occur with the side chains of serine or threonine residues in proteins.

Also, photolysis of azides in the presence of amines produce small yields of hydrazines which can be accounted for by insertion of the nitrene into the N-H bond of the amine. This product is probably produced via a primary attack by the nitrene on the non-bonding pair of the amino group followed by rearrangement (Hafner et al., 1964). Nevertheless, this insertion will give productive cross-linked product between the photoaffinity probe and the receptor.



$$R\text{-}NH_2 + {\uparrow\downarrow}\ddot{N}\text{-}R' \text{------}\!> \left[R\text{-}\underset{H}{\overset{H}{N}}\text{-}\underset{\cdot\cdot}{\overset{\uparrow\downarrow}{N}}\text{-}R' \right] \text{-----}\!> R\text{-}\overset{H}{N}\text{-}\underset{H}{N}\text{-}R'$$

b. CYCLOADDITION REACTION

Addition to double bonds is a reaction of both singlet and triplet nitrenes, and consequently produces aziridenes (Fig. 4.1, pathway c). The addition of the singlet nitrene proceeds by a one step mechanism and the aziridine retains the steric configuration of the olefinic substrate. Triplet nitrenes add to double bonds with retention of configuration. The yields for the incorporation of photolabel into the fatty acids is around 3% (Bayley and Knowles, 1978).

Doering and Odum(1966) also observed formation of azepines on irradiation of phenyl azide in the presence of strong nucleophiles (Fig. 4.1, pathway e). This could happen to the side chain of lysine in proteins.

c. H ABSTRACTION AND COUPLING

Hydrogen abstraction is possibly the most general reaction of triplet nitrenes. Two separate abstraction steps are required to saturate the electron deficiency of the triplet. In the first step hydrogen is abstracted from the substrate leaving a carbon radical behind and turning the nitrene into an amino-radical (Fig. 4.3, equation 1). The two radicals at this stage have the

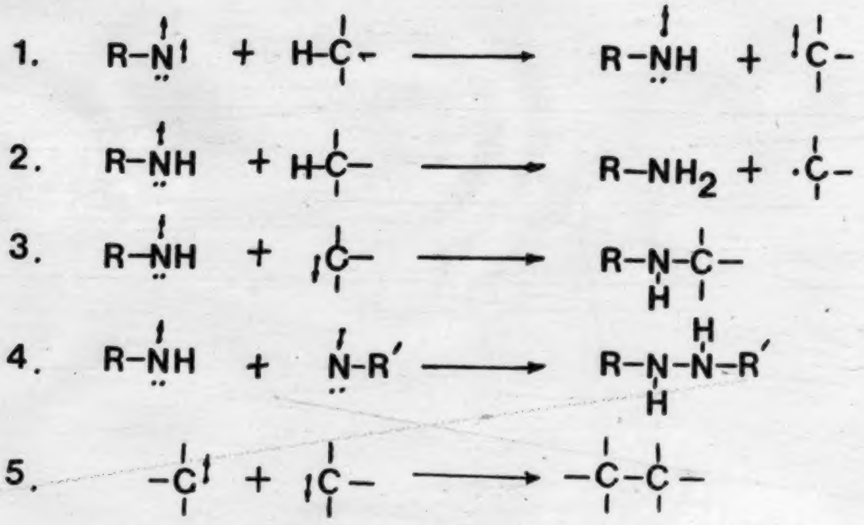


Fig. 4.3 Reaction Mechanism of triplet nitrene in Hydrogen abstraction (for detail see text).

same unpaired spins and cannot couple unless one of their spins is reversed. The time required for spin reversal could allow the two radicals to diffuse away from each other. The amino-radical will then abstract a second hydrogen and form a primary amine (Fig. 4.3, equation 2). There is a certain probability of radical recombination leading to the secondary amine (Fig. 4.3, equation 3), hydrazines (equation 4), hydrocarbon dimers (equation 5). Equations 3-5 in Fig. 4.3 could generate productive covalent bond between the ligand and the receptor.

C. Properties of Ideal Heterobifunctional Cross-linking Reagents

Photoactivated cross-linking techniques have been extended to study quaternary structure (i.e. to determine the proximity of subunits to each other) in a multiple subunit biological system (Ji, 1974). For this purpose the cross-linking reagents are usually homo-bifunctional. However if one desires to determine the sites of interaction or spatial orientation between proteins in a multi-subunit system, then one must attach a cross-linker to a known position in one protein before cross-linking. Therefore, an ideal cross-linking reagent should be heterobifunctional so that it permits the following:

i) The two functional groups should be sufficiently different to permit well controlled sequential reactions of each group in turn;

ii) specific and selective incorporation into one protein in a benign medium;

iii) easy determination of the degree of incorporation;

iv) allow easy removal of excess reagents;

v) allow binding of the modified protein to the second protein or proteins and permit testing for the correct protein-protein interactions;

vi) non-specific cross-linking of the bound proteins without changing the biological condition of binding;

vii) and easy reversal of the cross-linking so that there is no ambiguity in identifying the site of interaction.

The two functional groups chosen to meet the above criteria were the 2-pyridyl disulfide moiety and the aryl azido moiety (Fig. 4.4). First, the 2-pyridyl disulfide group provides specific and selective attachment of the reagent to the protein SH group through the cysteine side chain of the reagent via a disulfide bridge. This reaction proceeds rapidly under mild condition in aqueous media and is not affected by pH (Brockleburst and Little, 1972). The release of the pyridine-2-thione during disulfide bridge formation can be followed spectrophotometrically at 343 nm (Carlesson et al.,1978) to provide a convenient method for controlling the degree of substitution. Second, the aryl azide as a photoaffinity probe has several advantages over conventional chemical labile reagents (see section A in Chapter IV). The aryl azide is inert until photolysis, permitting the removal of the excess bifunctional reagents from the modified protein and the control experiments to ensure the correct protein-protein interactions. The aryl azide is non-specific, in that it does not require the presence of a particular reactive functional group at the binding site for cross-linking to occur. The aryl azide is chemically stable at 37°C, is not drastically susceptible to photochemical rearrangement, and can be photolyzed to aryl nitrene at a wavelength above 320 nm to prevent damage to the protein (Galardy, 1973).

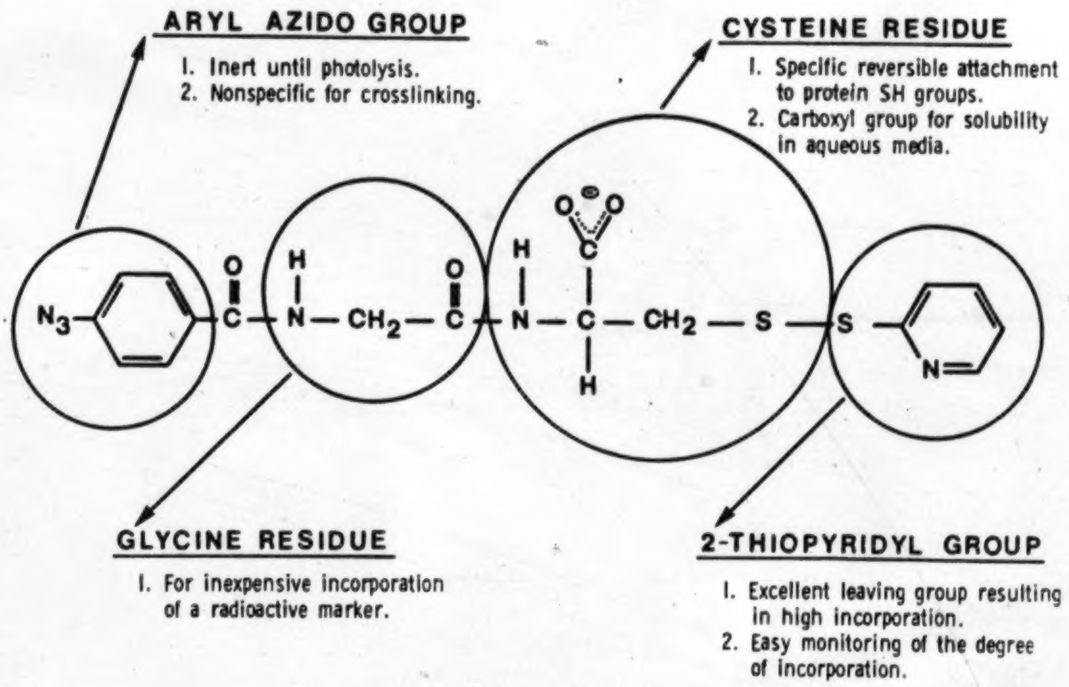

ARYL AZIDO GROUP
1. Inert until photolysis.
2. Nonspecific for crosslinking.
CYSTEINE RESIDUE
1. Specific reversible attachment to protein SH groups.
2. Carboxyl group for solubility in aqueous media.
GLYCINE RESIDUE
1. For inexpensive incorporation of a radioactive marker.
2-THIOPYRIDYL GROUP
1. Excellent leaving group resulting in high incorporation.
2. Easy monitoring of the degree of incorporation.
N3
O
C
H
N
CH2
O
C
H
N
O
O
C
C
H
CH2
S
S
N

Fig. 4.4. Stricture of the heterobifunctional photoaffinity probe: AGTC.

A general problem in working with bifunctional reagents is the low solubility of many of the available reagents in aqueous media used for studying protein-protein interaction. For this reason it is an advantage to incorporate a carboxylate function into the reagent which would be ionized at most pH's used to study protein-protein interactions. The addition of a radiolabel into the reagent is also useful for determining the degree of incorporation of the photoaffinity probe into the protein and most important in tagging the binding site on the second protein. For this purpose glycine was incorporated into the reagent as an inexpensive radiolabel. This reagent can be shortened by removing the glycine and incorporating the radiolabel elsewhere. Therefore both reagents can be used to monitor the conformational changes in multi-subunit biological systems.

D. Chemical and Physical Analytical Methods

1. Physical Methods

Melting points which were determined in an electrothermal melting point apparatus are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded with either a Varian Model A60 or a 270 MHz Bruker spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield from the internal standard sodium 2,2dimethyl-2-silcapentane-5-sulfonate (dss). Elemental analyses and mass spectrometry were performed at the Micro-analytical Laboratory, Department of Chemistry, University of Alberta. Ultraviolet (UV) spectra were obtained using a recording spectrometer Cary Model 118C.

2. Chemical Methods

Thin layer chromatography was performed with precoated silica gel plates with a fluorescent indicator obtained from EM Laboratories Inc. (60F-254). The following solvent systems were used:

System A-- 1-butanol/acetic acid/water,7:2:1;
System B-- 1-butanol/acetic acid/water,4:1:5 top phase;
System C-- Chloroform/methanol/acetic acid,85:10:5;
System D-- Chloroform/methanol,1:1.

The compounds were visualized directly under 254 nm UV light or by spraying with ninhydrin in acetone (1%) followed by heating. The compounds were homogeneous in the solvent systems as described above, and 10-100 ug of compound was applied to the thin layer plate.

3. Determination of the Degree of Incorporation of the Photoaffinity Probes into Proteins

The incorporation of the arylazide moiety was followed by the release of pyridine-2-thione which has a molar absorptivity of 8.08x10^3 at 343 nm (Carlesson et al.,1978) during the thiol disulfide exchange. After removal of excess reagent by gel filtration, the incorporation was determined by the absorptivity of the modified protein at 230 nm and 270 nm in accordance with the following equations:

$$A^{M}_{230} = C_{P}E^{P}_{230} + C_{R}E^{R}_{230}$$

$$A^{M}_{270} = C_{P}E^{P}_{270} + C_{R}E^{R}_{270}$$

$$I_R = {}^{C_R}/_{C_P} = \frac{A^{M}_{270} \times E^{P}_{230} - A^{M}_{230} \times E^{P}_{270}}{A^{M}_{230} \times E^{P}_{270} - A^{M}_{270} \times E^{R}_{230}}$$

Where A^M is the absorbance of the modified protein; C_R and C_P are the molar concentrations of reagent(AGC) and protein respectively; E^R and E^P are the molar absorptivities of the reagents (AGC) and the protein, respectively; and I_R is the incorporation of reagent per mole of protein. The molar absorptivities of reagents and proteins are given in Table 4-III. When radioactive AGTC (N-(4-azidobenzoyl[2-^{3}H]glycyl-S-(2thiopyridyl)-cysteine and ATC (N-(4-azido[1-^{14}C]benzoyl-S-(2-thiopyridyl)-cysteine) were used, the incorporation was determined from the concentration of protein as determined by amino acid analyses and radioactivity measurements.

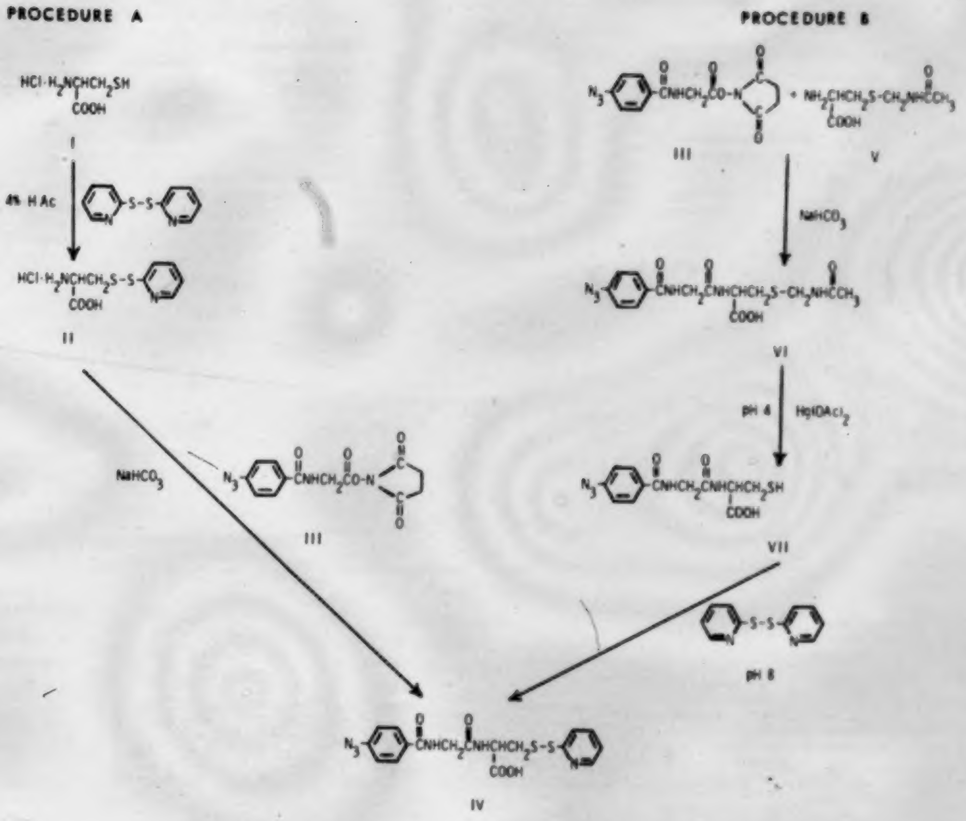

PROCEDURE A
PROCEDURE B
I
4% HAc
II
NaHCO3
III
V
VI
pH 4
Hg(OAc)2
VII
pH 8
IV

Fig. 4.5. Scheme for the Synthesis of the Heretobifunctional Photoaffinity probe AGTC.

E. Synthesis And Chemical Characterization Of AGTC

1. Organic Synthesis-- Procedure A

Organic synthesis of AGTC has been summarized in Fig. 4.5.

a. 2-PYRIDYLDITHIOCYSTEINE HYDROCHLORIDE (II)

Cysteine hydrochloride (I) (1.58 g, 10 mmole) was dissolved in 10 ml of methanol and added dropwise into a solution of 2,2'dithiopyridine(4.4 g, 20 mmole) in 20 ml of methanol containing 0.8 ml of acetic acid with vigorous stirring. The reaction mixture was stirred for 24 hr at room temperature and evaporated to dryness to give an oil. The oil was triturated with 50 ml of dichloromethane and evaporated to dryness. The residue was dissolved in a small volume of methanol and anhydrous ether was added to precipitate the product. The precipitate was washed with ether and dried under high vacuum over KOH pellets. Yield: 2.08 g (78%), m.p. 158-159°C, R_f 0.27 in system B. NMR analysis in CD_3OD (Varian A60) showed δ 2.31(multiplet, 2H, CH_2), 3.18(quartet, 1H, J=3.4 Hz, CH), 6.57(multiplet, 3H, aromatic), 7.53(d, 1H, J=4.3 Hz, aromatic). Elemental analysis for $C_8H_{11}N_2O_2S_2Cl$:

	C	H	N	S	Cl
Calculated	36.02	4.16	10.50	24.04	13.29
Found	35.78	4.35	10.39	24.15	12.65

TABLE 4-I

Extinction Coefficient and Molecular Weights

Proteins or Reagents[a]	Molecular Weights	Molar Absorptivity 230 nm (x1000 $M^{-1}cm^{-1}$)	270 nm
TnC	18,000	42.5	2.35
TM	33,000[b]	91.4	8.74
AGTC	450		26.2
ATC	375	11.0	22.0
AGC	323	2.76	15.0
AGC[c]		6.79	3.71

[a] TnC, rabbit skeletal troponin C; TM, Rabbit cardiac tropomyosin.

[b] Subunit molecular weight.

[c] After photolysis.

b. 4-AZIDOBENZOIC ACID

This compound was prepared essentially according to Galardy(1973). To a stirred suspension of 4-aminobenzoic acid(66.4 g, 0.48 mole) in 750 ml of water and 100 ml of concentrated sulfuric acid cooled in an ice bath was added sodium nitrite(40.25 g, 0.60 mole) in 375 ml of water over a period of 40 min. As soon as the suspension dissolved, urea(5 g) was added with vigorous stirring to destroy excess nitrous acid. After 30 min, sodium azide(64.3 g, 0.99 mole) in 300 ml water was slowly added with continuous stirring over a period of 2 hr. After evolution of nitrogen ceased, the mixture was stirred for another 1 hr. The precipitate was filtered off and washed with ice water, crystallized from 95% ethanol and dried under high vacuum over P_2O_5. The product was recrystallized from 50% ethanol/ water(V/V) to give 64.0 g of 4-azidobenzoic acid. Yield 83%, m.p. 182-183d, R_f 0.95 in system B. IR analysis(nujol) confirmed the arylazido group at 2105 cm^{-1}.

c. N-HYDROXYSUCCINIMIDE ESTER OF 4-AZIDOBENZOIC ACID

This compound was prepared as previously described by Galardy (1973) except the reaction time was increased from 12 to 20 hr and the final recrystallization from dioxane-anhydrous ether to yield 4.2 g, 82%, m.p. 173-174°C, R_f 0.84 in system B.

d. 4-AZIDOBENZOYLGLYCINE

N-hydroxysuccinimide ester of 4-azidobenzoic acid (6.23 g, 28.3 mmole) in 200 ml of dioxane was added to a cold solution of $NaHCO_3$ (3.9 g, 46.4 mmole) and glycine (1.75 g, 23.3 mmole) in 100 ml of water. After 24 hr with constant stirring at room temperature, the reaction mixture was concentrated to 60 ml, cooled in an ice bath and the pH adjusted to 2.5 with cold 1N HCl. The precipitate was collected and recrystallized from water to give 3.23 g, 63% yield. m.p. 132-133°C, R_f 0.80 in system B and 0.46 in system D. NMR analysis in D_2O (270 Bruker) showed δ 7.85(doublet, 2H, aromatic), 7.25(doublet, 2H, aromatic), 4.10(singlet, 2H, CH_2 of glycine). The mass spectrum showed a parent peak at 220.06 and a base peak at 135.03.

e. N-HYDROXYSUCCINIMIDE ESTER OF 4-AZIDOBENZOYLGLYCINE(III)

Dicyclohexylcarbodiimide (1.05 g, 5.1 mmole) in 2 ml of dioxane was added into a solution of 4-azidobenzoylglycine (1.12g, 5.1 mmole) and N-hydroxysuccinimide (0.71 g, 5.1mmole) in 15 ml of dioxane in an ice bath. After 20 hr at room temperature with constant stirring the solution was filtered and evaporated to dryness. The product was crystallized twice from isopropanol-dioxane to give 0.93 g, 57% yield, m.p. 167-168°C, R_f 0.75 in system B and 0.98 in system D. The mass spectrum showed the parent peak at 317.08 and the base peak at 115.03. NMR analysis(Varian A60) in CD_3CO showed δ 2.89(singlet, 2H, CH_2 of N-hydroxysuccinimide), 3.62(singlet, 2H, CH_2 of N-hydroxysuccinimide), 4.52(doublet, 2H, J=10 Hz, CH_2 of glycine), 7.25(doublet, 2H, J=15 Hz, aromatic), 8.05(doublet,2H, J=15 Hz, aromatic), 8.15(multiplet, 1H, NH).

f. N-(4-AZIDOBENZOYLGLYCYL)-S-(2-THIOPYRIDYL)-CYSTEINE (IV)

N-hydroxysuccinimide ester of 4-azidobenzoylglycine (1.189 g, 3.75 mmole) dissolved in 40 ml of dixoane was added slowly into 10 ml of water containing 2-pyridyldithiocysteine hydrochloride,II (1.0 g, 3.75 mmole) and sodium bicarbonate (0.63 g, 7.5 mmole). The reaction mixture was stirred at room temperature for 24 hr and concentrated to 20 ml. To the reaction mixture in an ice bath,20 ml of cold water was added and the solution acidified with cold 1N HCl to pH 3.0. The white precipitate was dried over P_2O_5 under high vacuum to give 1.03 g (61% yield), m.p. 94-95°C, R_f 0.53 in system B and 0.26 in system C. NMR analysis (270 Bruker) in D_2O/ NaOH showed δ 3.21, 3.40(quartet, 2H, J=10 Hz, CH_2 of cysteine), 4.13(singlet, 2H, CH_2 of glycine), 4.52(quartet, 1H, J=5 Hz, CH of cysteine), 7.28(multiplet, 3H, aromatic), 7.85(multiplet, 4H, aromatic), 8.45(doublet, 1H, J=5 Hz, C^6H of pyridine). IR analysis(nujol) confirmed the aryl azido group at 2105 cm^{-1}. Elemental analysis for $C_{17}H_{18}N_6O_5S_2$:

	C	H	N	S
Calculated	45.35	4.02	18.67	14.24
Found	45.77	3.98	18.37	13.40

g. ^3H-AGTC

Five millicurie(0.33 mmole) of [2-^{3}H]glycine (New England Nuclear) in 5 ml of 0.1N HCl was neutralized to pH7.5 with 1N NaOH. To this solution was added cold glycine (1.0 g, 13.31 mmole), $NaHCO_3$ (2.27 g, 26.62 mmole) and 60 ml of water followed by N-hydroxysuccinimide ester of 4-azidobenzoic acid (3.75 g, 13.31 mmole) in 100 ml dioxane. The synthesis was continued as described above to yield 2.27 g of radioactive compound IV with a specific activity of 274 cpm/nmole. Similarly, a higher specific activity (2100 cpm/nmole) AGTC was synthesized from 25 millicurie [2-^{3}H] glycine.

h. ^{14}C-AGTC

Ten millicurie of 4-amino-[1-^{14}C]-benzoic acid (ICN Chemical and radioisotope Div. Irvine, California) was mixed with 445.38 mg of 4-aminobenzoic acid and dissolved in 6 ml of 1N H_2SO_4, then was diazotized to form 4-azido-[1-^{14}C]-benzoic acid. Syntheses were continued as described above except a two fold excess of glycine was used to increase the yield of 4-azido[1-^{14}C]benzoylglycine. 762.32 mg final product, ^{14}C-AGTC was obtained with a specific radioactivity of 5000 cpm per nmole.

2. Organic Synthesis-- Procedure B

a. N-(4-AZIDOBENZOYLGLYCYL)-S-(ACETAMIDOMETHYL)-CYSTEINE (VI)

N-hydroxysuccinimide ester of 4-azidobenzoylglycine (III) (1.0 g 3.15 mmole) in 40 ml of dioxane was added into a cold solution of S(acetamidomethyl)-cysteine(0.60 g, 3.15 mmole) which was prepared according to Veber et al.(1972) and sodium bicarbonate (0.67 g, 8.0 mmole) in 20 ml of water. The reaction mixture was stirred at room temperature for 16 hr, concentrated to 25 ml and acidified to pH2.5 with cold 1N HCl. The precipitate which was collected by filtration gave 0.94 g (76% yield). An analytical sample was crystallized from 95% ethanol and dried under high vacuum over P_2O_5, m.p. 184-185°C, R_f 0.53 in system B and 0.10 in system C. NMR analysis (Burker 270) in D_2O/NaOD showed δ 2.0(singlet, 3H, CH_3), 2.90, 3.10(quartet, quartet, 2H, J=10 Hz, J=5 Hz, CH_2 of cysteine), 4.05(singlet, 2H, CH_2 of glycine), 4.20(quartet, 1H, J=5 Hz, CH of cysteine), 4.45(quartet, 2H, J=6 Hz, CH_2 of acetamido), 7.25(doublet, 2H, J=10 Hz, aromatic), 7.85(doublet, 2H, J=10 Hz, aromatic).

Elemental analysis for $C_{15}H_{18}N_6O_5S$:

	C	H	N	S
Calculated	45.68	4.57	21.32	8.12
Found	45.67	4.56	21.07	7.37

b. N-(4-AZIDOBENZOYLGLYCYL)-CYSTEINE (VII)

Compound VI (420 mg, 1.07 mmole) was dissolved in 30 ml of 0.1N NaOH and the pH lowered to 4.0 with dilute acetic acid. Mercuric acetate (0.67 g, 2.1 mmole) was added into the solution of compound VI with stirring at room temperature. After 2 hr, the precipitate was collected by filtration and dried under vacuum over P_2O_5. The precipitate was resuspended in 5% acetic acid(40 ml) and hydrogen sulfide gas was passed through the suspension for at least 3 min. The reaction mixture was allowed to stand at room temperature for 1 hr and then filtered. The filtrate was extracted three times with 10 ml of ethyl acetate. The combined organic phase was washed with 20 ml of cold 1 mM HCl, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to give 139 mg (40%) of product (compound VII), m.p. 149-150°d, R_f 0.68 in system B and 0.15 in system C. NMR analysis (Bruker 270) in D_2O/NaOH showed δ 2.75, 2,95 (quartet,quartet, 2H, J=10 Hz, J=5 Hz, CH_2 of cysteine), 4.05 (singlet, 2H, CH_2 of glycine), 4.25(quartet, 1H, J=6 Hz, CH of cysteine), 7.25 (doublet, 2H, J=10 Hz, aromatic), 7.85 (doublet,2H, J=10

Hz, aromatic). IR analysis (nujol) confirmed the aryl azido group at 2105 cm^{-1} and the sulfhydryl group at 2720 cm^{-1}. Elemental analysis for the dicyclohexylamine salt $C_{24}H_{36}N_6O_4S$:

	C	H	N	S
Calculated	57.10	7.10	16.60	6.30
Found	56.03	6.94	16.32	6.17

c. N-(4-AZIDOBENZOYLGLYCYL)-S-(2-THIOPYRIDYL)CYSTEINE (IV)

To 0.5 ml solution of compound VII (30 mg, 92.9 umole), 2,2'dithiopyridine (40.94 mg, 185,8 umole) and $NaHCO_3$ (15.58 mg, 185.8 umole) was added. The reaction mixture was stirred at room temperature for 6 hr. TLC of the reaction mixture in solvent system B indicated the formation of compound IV with identical mobility to compound IV prepared by procedure A (R_f 0.53). No further characterization of the product was performed since compound IV can be prepared more simply by procedure A.

3. Chemical Characterization

a. CHEMICAL STABILITY OF AGTC

AGTC is a white solid with no tendency for deliquesence. Due to the presence of the azido moeity, solid AGTC was not stable to exposure to laboratory fluorescent light(Table 4-II). Therefore, the reagent was stored in the dark in a brown bottle wrapped with Al foil. No degradation of the reagent was observed after 1

TABLE 4-II

Chemical Stability of AGTC

	Treatments			Degradation	
[Reagent] mM	Buffer or Solvent[a]	Temp (C)	Time (hr)	Disulfide bridge[b]	Arylazido group[c]
0.09 to 11[d]	pH3.0, 20mM NaAc	22	48	none	none
	pH5.1, 20mM NaAc			none	none
	pH7.5, 0.1M NaHPO			none	none
	pH9.0, 0.1M NaHPO			none	none
44	pH7.5, 0.1M NaHPO	22	8	Partially[e]	none
44	Methanol	22	48	none	none
0.9	pH7.5, 0.1M NaHPO Laboratory Fluorescent light	22	168	none	none
	pH7.5, 0.1M NaHPO Irradiated at 350 nm	32	2	none	destroyed
Solid	Laboratory Fluorescent light	22	4	none	partially[f]

[a] All glassware containing solutions of AGTC were wrapped with tin foil to prevent exposure to light except when light exposure was desired.

[b] Determined spectrophotometrically by the release of pyridine-2-thione at 343 nm and by TLC in solvent system B.

[c] Determined spectrophotometrically by the decrease in absorbance at 270 nm and by TLC in solvent system B.

[d] Disulfide bridge and aryl azido stability studies were carried out at 11 and 0.09 mM at pH 3.0 and 5.1. The reagent was first dissolved in methanol and an aliquot added into the buffer to give the final reagent concentration.

[e] Partially degradation refers to less than 10% by spot intensity on TLC in solvent system B.

[f] Partially degradation refers to less than 10% as determined from the decrease in absorbance at 270 nm.

yr as tested by thin layer chromatography and the release of pyridine-2-thione. At room temperature AGTC ready dissolved in 0.1M Na_2HPO_4 buffer, pH7.5. The disulfide moiety was stable in solution at concentrations below 11 mM in the pH range from 3.0 to 9.0 over a period of 48 hr at room temperature (Table 4-II). However, at high concentration in aqueous solution (44 mM) AGTC was partially degraded (less than 10% in 8 hr as determined by the formation of pyridine-2-thione on TLC). This result is not surprising since the thiopyridyl group is a very good leaving group, resulting in intrathiol-disulfide exchange (Brocklehurst and Little, 1972; Carlsson et al.,1978). No degradation was observed at a concentration of 44 mM reagent in methanol (Table 4-II). The degradation of the reagent by intrathiol-disulfide exchange would not interfere with the thiol-disulfide exchange between AGTC and the SH groups in proteins because of the large excess of reagent. Also, the conditions used for protein modification in aqueous solution (see below) which involved a concentration less than 44 mM and a reaction time of 2 hr showed no reagent breakdown. The disulfide moiety is stable to the conditions of photolysis used to activate the azido group (Table 4-II). The aryl azide moeity is stable in aqueous media even at high reagent concentration. No degradation of the aryl azide moiety was observed when an AGTC solution was exposed to laboratory fluorescent light for 1 week at room temperature (Table 4-II). This property of AGTC should provide sufficient time to permit easy removal of excess reagents and to test for the correct protein-protein interaction of the modified protein in complex with other proteins.

To prevent the possible destruction of sensitive sulfur or aromatic amino acid residues in proteins during the photolysis, cross-linking experiments could be carried out using an irradiation source of 350 nm light. Galardy(1973) has shown previously that, with irradiation at a wavelength of 320 nm and above, neither sulfur-containing nor aromatic amino acids are affected by photolysis. This agrees with our results that no disulfide bridge degradation of the reagent was observed during photolysis for 2 hr (Table 4-II). Finally, the properties of high solubility and stability in aqueous media over a wide pH range make AGTC an ideal cross-linking reagent.

b. UV SPECTROSCOPIC PROPERTIES OF AGTC

Fig. 4.6 shows the UV spectrum of AGTC which has a maximum absorption at 270 nm with a molar absorptivity of 26.2 x 10^{3} $M^{-1}cm^{-1}$. In reducing medium pyridine-2-thione is released with a molar absorptivity of 8.08 x 10^{3} $M^{-1}cm^{-1}$ at 343 nm but this release does not alter the maximum absorption at 270 nm(Fig. 4.5). The UV

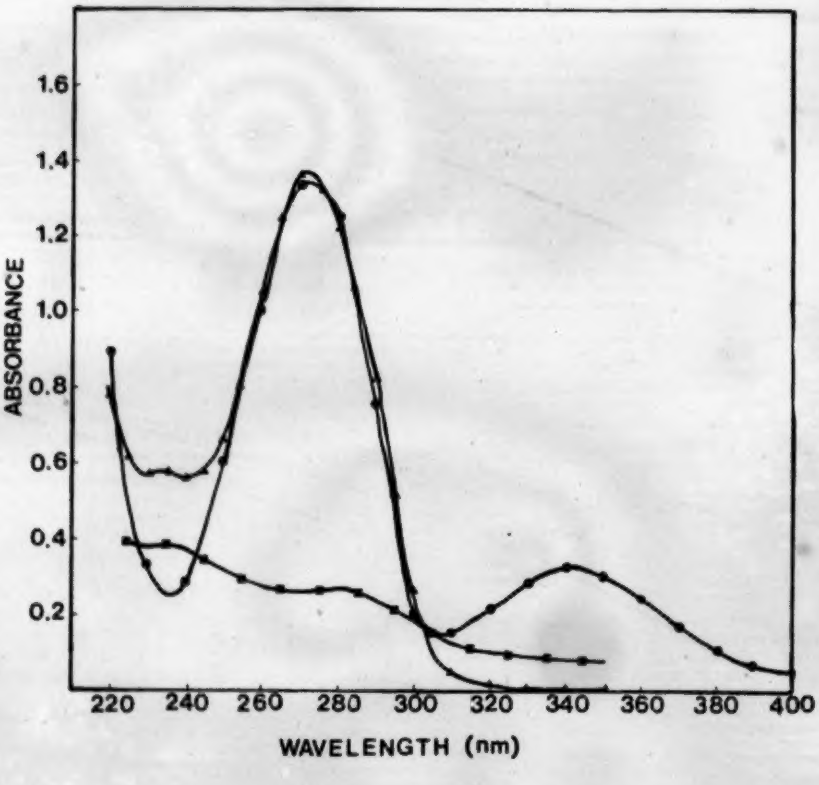


Fig. 4.6 Effect of photolysis and dithiothreitol treatment on the UV spectrum of the heretobifunctional photoaffinity probe, AGTC at a concentration of 50 μM in 50 mM Tris-HCl, 0.1 M KCl buffer, pH 7.5. UV spectrum of AGTC (▲—▲), after irradiation of 350 nM at 32 °C for 30 min (■—■), and after treatment with 25 mM dithiothreitol for 20 min (●—●).

spectrum of N-(4-azidobenzoylglycyl)cysteine (AGC, compound VII) in aqueous medium was similar to the UV spectrum of AGTC treated with reducing agent except for the peak shown at 343 nm due to pyridine-2-thione (Fig. 4.6).

Photolysis of AGTC for 30 min shows the loss of absorption at 270 nm due to destruction of the azido group(Fig. 4.6). The rate of decomposition of the aryl azide structure of AGTC during photolysis is similar in ethanol and aqueous media. Similarly, the photolysis of AGC-tropomyosin(Fig. 4.7) shows the complete destruction of the azido group in 10 min. No further change in the UV spectrum of the protein was observed after 2 hr of photolysis (Fig. 4.7). It is possible to calculate the amount of reagent attached to the protein after photolysis by using the absorption at 270 and 230 nm as previously described in this Chapter and the molar absorptivities of the reagent after photolysis (Table 4-I).

c. REACTIVITY OF AGTC

Since many proteins contain a small number of reactive SH groups usually involved in the sites of interaction, the feasibility of selective incorporation of one mole of AGTC per mole of protein via sulfhydryl attachment is possible. For example, rabbit skeletal and cardiac α-tropomyosin contain one cysteine residue at

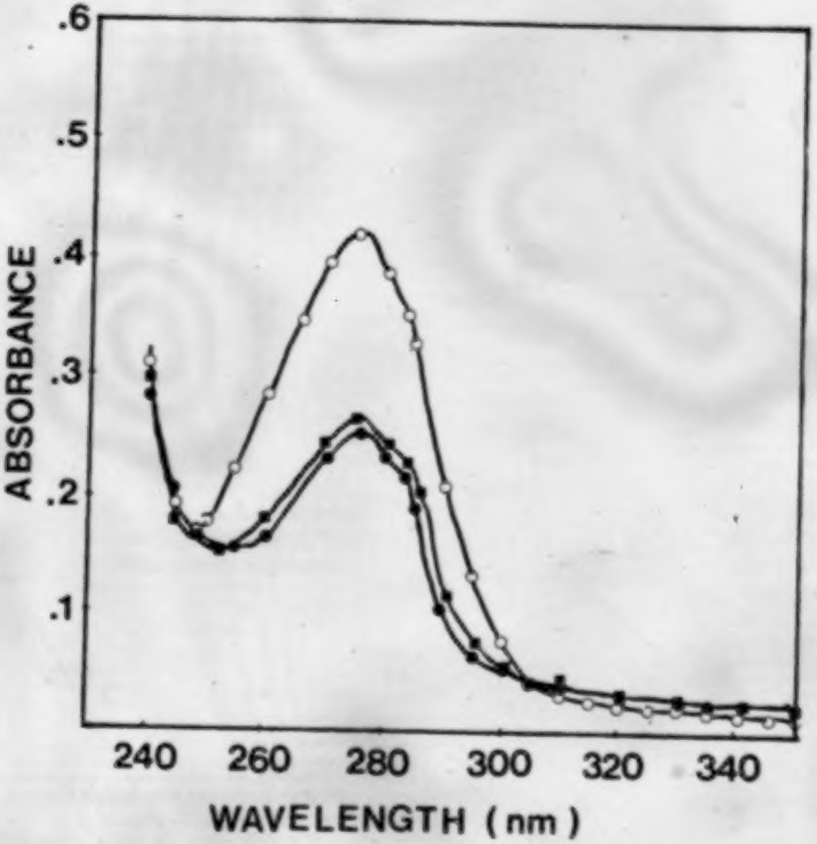


Fig. 4.7. Effect of photolysis on the UV spectrum of AGC attached to tropomyosin at cysteine 190 through disulfide bond formation (AGC-tropomyosin) at a concentration of 21 uM in 50 mM Tris-HCl, 0.1M KCl buffer, pH 7.5. Spectra are shown after irradiation at 350 nm and 32°C for 0 (o—o), 10 (■—■), and 20 (●—●) min. The 2 hr spectrum was identical with the spectrum obtained at 20 min.

position 190 (Stone and Smillie,1978), rabbit skeletal troponin C contains one cysteine at position 98 (Collin et al.,1973) and rabbit skeletal actin contains five cysteine residues. However cysteine 373 of actin can be specifically labelled (Elzinga et al.,1973). To demonstrate the reactivity of AGTC with protein SH groups, the above muscle proteins were modified. The results are summarized in Table 4-III and the introduction of the photoaffinity probe (aryl azide structure) into individual proteins is discussed below.

α-TROPOMYOSIN----The protein(6 mg) was dissolved in 2 ml of 10 mM Tris-HCl, 0.1M KCl, 10 mM DTT buffer, pH8.0 and dialyzed against the same buffer overnight at 4°C. This step ensured complete reduction of the free SH group of tropomyosin (TM). The DTT was removed from the protein by gel filtration on a Sephadex G-25 column (1x30 cm). To the freshly reduced protein solution (8 ml) was added 0.2 ml of a 15 mM solution of AGTC in 50 mM Tris-HCl, 0.1M KCl, buffer, pH7.5. The incorporation of the reagent was determined spectrophotometrically by the release of pyridine-2-thione. The degree of substitution can be varied by using different molar ratio of reagent to protein and incubation times (Table 4-III). A 92% incorporation into TM was achieved within 2 hr with an 85 fold molar excess of AGTC. The results in Table 4-III also indicate that there is good agreement in the methods used to determine the percent incorporation of AGTC into TM; that is, the measurement of the release of pyridine-2-thione at 343 nm and the calculation on the basis of absorption at 270 and 230 nm of the modified protein after the gel filtration using the equations described in this Chapter.

TROPONIN C----The protein (20 mg) was dissolved in 5 ml of 8M urea, 10 mM DTT, 1mM EGTA, 50 mM Tris-HCl buffer, pH7.5 and allowed to stand for 30 min to ensure that cysteine 98 was completely exposed and reduced. The protein solution was dialyzed against 500 ml of 50 mM Tris-HCl, 0.2M KCl, 1mM DTT,1 mM EGTA buffer, pH7.5 overnight at 4°C. The DTT was then removed by gel-filtration on a G-25 Sephadex column(1.5x100 cm) in the same buffer without DTT. To the protein fraction(14 ml) was added 76 mg of ^{3}H-AGTC (100 fold excess) in 0.5 ml of 1M Tris-HCl buffer, pH7.5. The reaction was monitored for the release of pyridine-2-thione at 343 nm. After 2 hr the release of pyridine-2-thione indicated the incorporation of one mole of AGTC per mole of protein. The reaction mixture was desalted as previously described to remove excess reagents. The modified troponin C was collected and the incorporation was measured using radioactivity measurements and amino acid analysis, and found to be >95%. The modified TnC solution was frozen and stored in a container covered with tin foil at -20°C. There was no loss of label from the modified TnC after 6 months of storage. Interestingly, there was only 30% incorporation of AGTC in TnC when the modification was carried out in the presence of calcium instead of EGTA. This suggests cysteine 98 of TnC is partially buried in the presence of calcium.

ACTIN----Actin (4 mg) in 2 ml of 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$ buffer, pH8.0 was dialyzed against the same buffer overnight at 4°C. The DTT was removed by gel filtration on a G-25 Sephadex column(1x30 cm). The actin solution was collected and polymerized into F-actin by adding a solution of 1M KCl and 1M $MgCl_2$ to give a final concentration of 50 mM KCL and 2 mM $MgCl_2$. A 100 fold excess of ^{3}H-AGTC was added to F-actin solution and stirred for 2 hr. The reaction mixture was then dialyzed against 4x4 litres of the above buffer without DTT for 2 days with nitrogen being bubbled through the dialyzate. The degree of incorporation was measured by radioactivity measurements and amino acid analysis. The radioactivity measurements showed 98% modification(assuming only cysteine 373 was labelled).

The above results suggest that AGTC can be readily incorporated into SH groups of proteins in benign media and the release of pyridine-2-thione allows easy

TABLE 4-III

Incorporation of AGTC into Proteins

Proteins	Reagent/protein Molar Ratio	% Incorporation with Time (hr)[a]			
		0.3	2	8	16
Tropomyosin	10.4	28	46	56	77(77[b])
	28.5	33	70	77(71[b])	
	85.4	38	92	82	
Troponin C	100		>95[b,c]		
Actin	100		>98[c]		

[a] All incorporations were determined by the release of pyridine-2-thione at 343 nm.

[b] Calculated on basis of absorbance at 270 and 230 nm of the modified proteins after the gel-filtration on a G-25 Sephadex column.

[c] Calculated on basis of radioactivity of the modified protein after the gel-filtration on a G-25 Sephadex column or dialysis to remove excess reagents.

spectrophotometric determination of the degree of incorporation.

d. REVERSIBILITY OF AGC-PROTEIN DISULFIDE LINK

To demonstrate the possibility of using AGTC for tagging and identifying the sites of interaction between proteins in a biological system, the reversibility of the disulfide bridge between AGC and α-tropomyosin was tested in the presence of reducing agent with and without irradiation of the protein (Table 4-IV). When AGC-tropomyosin was treated with a 6M urea, 0.1M DTT buffer overnight 92% of the reagent was removed from the sulfhydryl group of TM . This protein was isolated after reduction by gel-filtration as previously described to remove the urea and DTT and relabelled with AGTC (90 fold molar excess). The percent incorporation was 86% (Table 4-IV) indicating reversibility of the disulfide attachment. The stability of the disulfide bridge between reagent and protein to photolysis was shown in Table 4-IV. After 2 hr of irradiation, no new reagent could be incorporated into the protein unless DTT was added to reduce the disulfide bridge. After reduction and separation of the reduced protein by gel-filtration new reagent could be readily incorporated(Table 4-IV).

e. THE STRATEGY FOR THE USE OF AGTC

The results above suggest a general approach for identifying the interaction sites of proteins in close

TABLE 4-IV

Reversibility of Disulfide Bridge Between AGC and Tropomyosin

Treatment of Modified Proteins[a]	% Cleavage[b]	% Reincorporation of AGTC[c]
No irradiation 6M urea, 0.1M DTT	92	86
2 hr irradiation at 350 nm	-	0
2 hr irradiation at 350 nm followed by addition of 6M urea, 0.1M DTT	-	94

[a] The protein was dissolved in 50 mM Tris-HCl, 0.1M KCl buffer pH7.5 for photolysis on incorporation of AGTC. For cleavage of the disulfide bridge 6M urea and 0.1M DTT were included in the above buffer.

[b] Calculated on basis of absorbance at 270 and 230 nm.

[c] Determined by the release of pyridine-2-thione at 343 nm after 8 hr and 90 fold molar excess of AGTC.

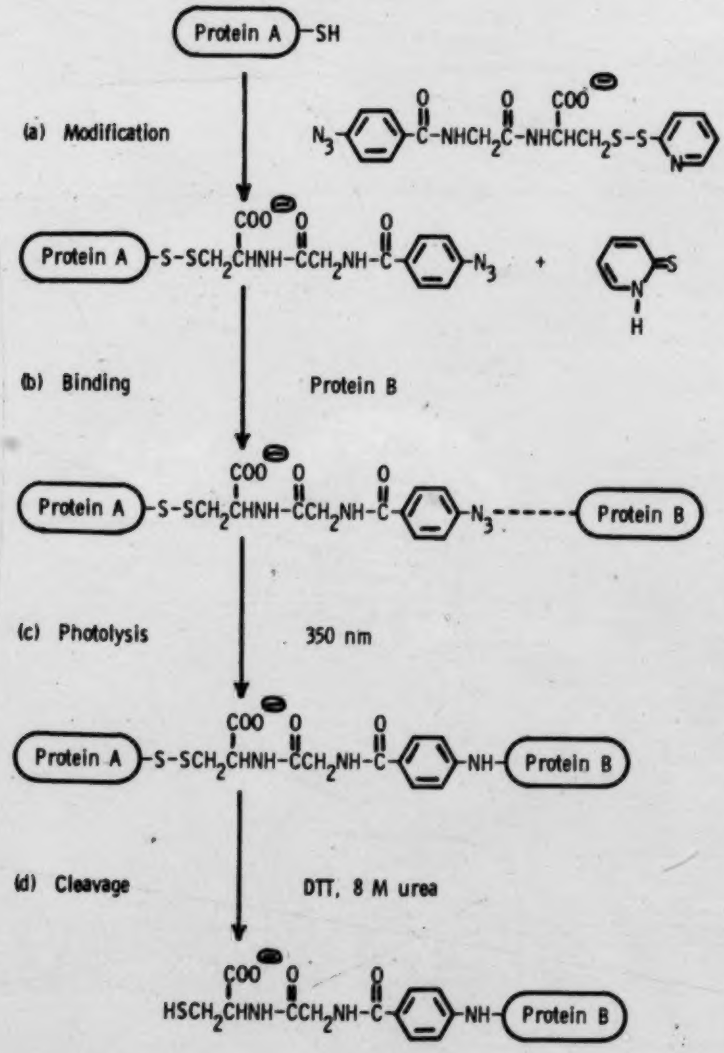

Protein A
SH
(a) Modification
(b) Binding
Protein B
(c) Photolysis
350 nm
(d) Cleavage
DTT, 8 M urea

Fig. 4.8. Scheme for the radiolabeling of a binding site on one protein which is in the vicinity of a SH group on another protein. The SH group is modified with the heretobifunctional photoaffinity probe AGTC. a, introduction of the aryl azide structure into a thioprotein A by thio-disulfide exchange; b, noncovalent binding of modified protein A to protein B; c, covalent cross-linking of the proteins by photolysis of the aryl azide moiety; d, cleavage of the disulfide bridge linking the two proteins with dithiothreitol, thus completing the transfer of radiolabel from protein A to protein B.

proximity to SH groups. Fig. 4.8 outlines the general strategy for the use of AGTC. First, protein A is reduced and AGTC is incorporated through disulfide bridge formation at cysteine residues. The degree of incorporation of AGC can be followed spectrophotometrically at 343 nm (Carlesson et al.,1978). The second step is the binding of modified protein A to protein B. The advantage of AGTC is that the aryl azido moiety is completely stable in aqueous media which allows the easy removal of excess reagent and testing for the correct protein-protein interaction with protein B. The third step is to form the covalently cross-linked complex between protein A and protein B by photolysis. To prevent the possible destruction of sensitive sulfur or aromatic residues in the proteins during photolysis, crosslinking is performed by using an irradiation source of 350 nm. In the absence of reducing agents, the cross-linked complex, protein B-AGC-protein A, can be isolated from any non-covalently cross-linked materials. The final step is to complete the transfer of the AGC group from protein A to protein B. This can be easily accomplished in the presence of reducing agents such as DTT to cleave the AGC-protein A disulfide bond to yield protein B-AGC. Using radioactive AGTC, the protein B-AGC can be isolated and used for identifying the residues involved in the site of interaction between the two proteins. The application of AGTC to tag the binding sites on TnI for TnC, TnT for TnI and troponin for tropomyosin have been successfully carried out (see Chapters V, VI, VII).

F. Synthesis And Chemical Characterization Of ATC

1. Organic Synthesis

a. N-(4-AZIDOBENZOYL)-S-(2-THIOPYRIDYL)CYSTEINE (ATC)

Sodium bicarbonate (0.97 g, 10.59 mmole) dissolved in 15 ml distilled water was slowly added into 15 ml of water containing S-(2-thiopyridyl)cysteine hydrochloride (1.36 g, 50.40 mmole). The solution was mixed for 3 min, then was slowly added into a cold solution containing N-hydroxysuccinimide ester of 4-azidobenzoic acid(1.23 g, 47.27 mmole per 40 ml dioxane) over a period of 5 min. The reaction mixture was stirred at room temperature for 20 hr and then concentrated to 15 ml on a rotary evaporator. Cold 1 mM HCl (30 ml) was added to the reaction mixture in an ice bath and the solution was acidified with cold 1N HCl to pH3.0. The white precipitate was collected by filtration and washed 2 times with 10 ml cold 1 mM HCl. The precipitate was crystallized from 50% ethanol and then recrystallized again from benzene. The crystals were dried over P_2O_5 under high vacuum. Yield: 1.143 g; 64.5%; m.p. 134-136°C; R_f= 0.55 in system A. NMR analysis (270 Bruker) in D_6-acetone showed δ 3.42(multiplet, 2H, CH_2 of cysteine), 4.93(multiplet, 1H, CH of cysteine), 7.15(multiplet, 3H, aromatic), 7.74(multiplet, 2H, aromatic), 7.93(multiplet, 3H, aromatic), 8.20(doublet, 1H, J=4 Hz, C^6H of pyridine). IR analysis(nujol) confirmed the aryl azido group at 2105 cm^{-1}. Elemental analysis for $C_{15}H_{13}O_3N_5S_2$:

	C	H	N	S
Calculated	47.99	3.49	18.65	17.08
Found	47.58	3.44	18.38	17.25

b. N-(4-AZIDO[1-^{14}C]BENZOYL)-S-(2-THIOPYRIDYL)CYSTEINE

Five mCi (22.85 mg) of 4-amino-[1-^{14}C]benzoic acid (ICN Chemical and radioisotope Div. Irvine, California) was mixed with 1.035 g 4-aminobenzoic acid and dissolved in 14 ml of 1N H_2SO_4, then was diazotized to form 4-azido-[1-^{14}C]benzoic acid. The syntheses were continued as described above to give 1.20 g of N-(4-azido[1-^{14}C]benzoyl)-S-(2-thiopyridyl)- cysteine with a specific radioactivity of 1550 cpm per nmole.

V. BIOCHEMICAL APPLICATION OF AGTC TO STUDY TnI-TnC INTERACTION

A. TnI-TnC Interaction

In Chapter III, it has been shown that cysteine 98 of rabbit skeletal troponin C was protected by troponin I from S-carboxamidomethylation with iodoacetamide. Also it has been mentioned that a CNBr fragment of TnC, CB9(residues 84-135) is able to inhibit the phosphorylation at serine 117 of rabbit skeletal TnI by protein kinase and unlike TnC, was much less effective in inhibiting phosphorylation by phosphorylase kinase at threonine 11 of TnI (Perry,1979). Two mechanisms have been proposed by Perry(1979) to explain how the interaction of TnC affects two regions of TnI molecule. One hypothesis is that the two TnC interacting sites are so close that once the TnI-TnC complex is formed both are effectively blocked by TnC. The other hypothesis is that TnC sterically blocks one site (serine 117) and causes conformational changes within TnI that render the second site (threonine 11) unavailable for phosphorylation. Thus, studies which can determine the residues involved in the interaction sites will be invaluable in distinguishing between these hypotheses and also will lead to the understanding of the function of the whole troponin complex in the regulation of muscle contraction and relaxation.

We feel that the best method for determining the residues involved in the sites of interaction between proteins is to use bifunctional cross-linking reagents. In Chapter IV, I have discussed the synthesis and characterization of a heterobifunctional photoaffinity probe, AGTC. Since cysteine 98 of TnC has been shown to be in the site of interaction with TnI and AGTC can be specifically attached to this cysteine residue (see Chapter IV), one should be able to identify the sites of interaction between TnC and TnI in proximity to cysteine 98. The strategy of the use of AGTC has been outlined in Chapter IV, so in this Chapter I will describe the biochemical application of AGTC in identifying the site of interaction between bovine cardiac TnI and rabbit skeletal TnC. The reason for using such hybrid system is that the AGTC modified TnC binds much tighter to the alkylated bovine cardiac TnI than to the alkylated rabbit skeletal TnI (see the results below).

B. Experimental Procedures

1. Conditions for Noncovalent Complex Formation between TnI and TnC

In complex formation experiments between rabbit skeletal TnC and bovine cardiac TnI, rabbit skeletal AGC-TnC and bovine cardiac TnI, and rabbit skeletal AGC-TnC and bovine S-carboxamidomethylated TnI, the following method was used: To 1.12 umole of radioactive AGC-TnC in 3 ml of 100 mM Tris-HCl, 0.2M KCl buffer, pH7.5 was added 100 ul of 1M $CaCl_2$ solution. To this stirring solution of TnC was added dropwise a solution (20 ml) of bovine cardiac TnI (56nmole per ml) in 1 mM HCl, 0.2M KCl over a 3 min period. The protein mixture was stirred under nitrogen at 4°C for 1 hr. The reaction vessel was wrapped with tin-foil to prevent any undesired reaction from the laboratory light when AGC-TnC was used. Also, the reason for using 2:1 molar ratio of TnC to CM-TnI is due to the fact that CM-TnI is not soluble at low ionic strength at pH7.5 and we want to have complete TnC-CM-TnI complex formation.

2. Other Methods

Protein preparations, protein modifications, amino acid analyses, DEAE-A50 Sephadex chromatography and SDS-Urea polyacrylamide gel electrophoresis were carried out as described in Chapter II.

C. Results

1. The Stability of TnI-TnC Complex

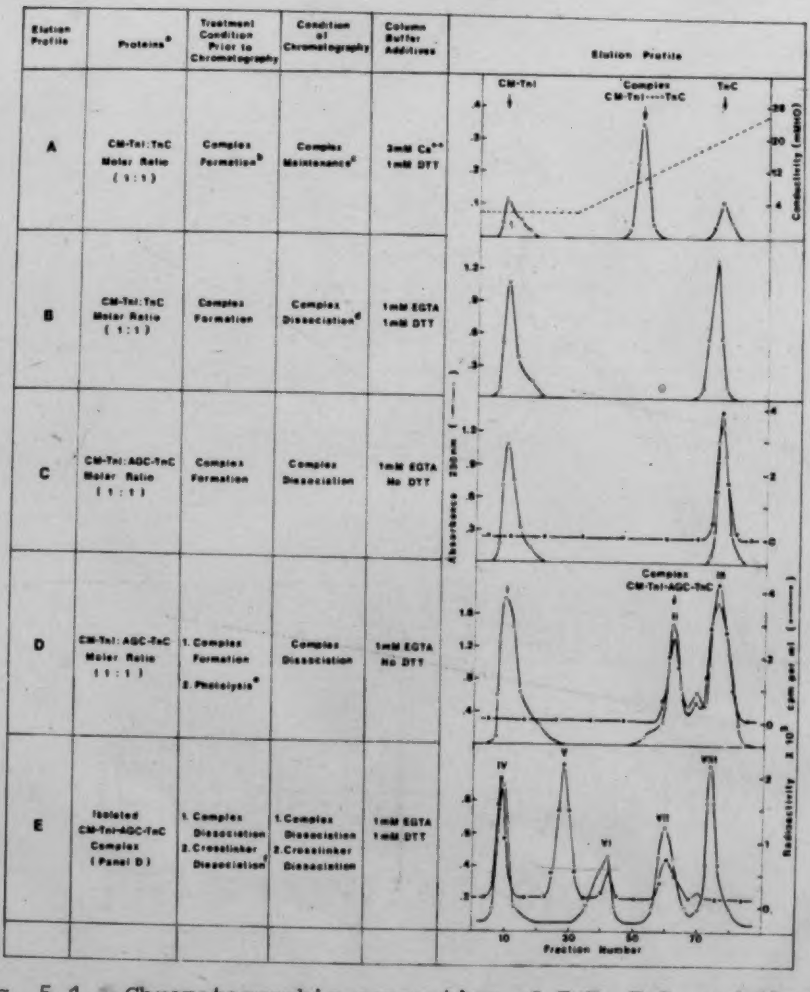
In Chapter III, we have discussed the importance of maintaining the SH groups of the troponin subunits in the reduced state for producing a troponin complex which is both biologically active and responsive to conformational changes. Therefore, in the study of TnI-TnC interaction the SH groups must be protected to prevent any non-physiological disulfide formation (both intra and inter-molecular). In this study, the heterobifunctional photoaffinity probe, AGTC is attached to cysteine 98 of TnC via the disulfide bridge. Because of this, dithiothreitol (DTT) cannot be used to maintain the SH groups of TnI in the reduced state. The fully reduced TnI can be isolated after dialysis against DTT and rapid gel-filtration and maintained in the reduced state by purging the buffers with nitrogen for avoiding air-oxidation of the SH groups. However, to avoid any possible complications of air-oxidation of TnI we decided to protect the SH groups of TnI by S-carboxamidomethylation.

Head and Perry(1974) first showed the extreme stability of the TnI-TnC complex in the presence of calcium. Using polyacrylamide gel electrophoresis the complex was shown to be stable in 8M urea and in the pH range 7 - 8.6. Also TnI cannot be eluted from a TnC-Sepharose affinity column in the presence of calcium with increasing ionic strength from 0 to 0.5M KCl unless EGTA is added to the eluting buffer. Similarly, Greaser and Gergely(1973) showed that the troponin complex could be separated into its components(TnI, TnT, TnC) on a DEAE-A50 Sephadex column in 6M urea and 1 mM EGTA. Considering the extreme stability of TnI-TnC complex it seemed possible that DEAE-A50 Sephadex chromatography could be used to isolate the TnI-TnC complex in the presence of calcium from unbound TnI and TnC.

In our first experiment, we found the complex formed from unmodified TnC and unmodified rabbit skeletal TnI was stable to DEAE-Sephadex chromatography in the presence of calcium-6M urea. Unfortunately, the complex formed from the unmodified TnC and the S-carboxamidomethylated rabbit skeletal TnI was separated into its components on the DEAE-Sephadex column even in the presence of calcium. These results indicated the S-carboxamidomethylation of rabbit skeletal TnI had somehow weakened the interaction between TnC and TnI. In Chapter III, we found that cysteine residues 48 and 133 of rabbit skeletal TnI were partially protected by TnC in the presence of Ca^{2+}. The reduced binding affinity between TnC and S-carboxamidomethylated TnI could be due to the steric hindrance at the region around cysteine residues 48 and 133.

Hincke et al.(1977) have shown that the components of the regulatory system of bovine cardiac and rabbit skeletal muscles can form hybrid complexes, which still maintain the biological activities. Further studies(Hincke et al.,1979) showed that the interaction of the bovine cardiac TnC and the S-carboxamidomethylated bovine cardiac TnI was similar to that of the native proteins. Therefore, we carried out an experiment to test if a complex formed from rabbit skeletal TnC and S-carboxamidomethylated bovine cardiac TnI would be stable to DEAE-Sephadex chromatography in the presence of calcium. To form the complex bovine cardiac S-carboxamidomethylated TnI (CM-TnI) was mixed with rabbit skeletal TnC in a 1:1 molar ratio and stirred for 1 hr at room temperature in benign buffer as described in the "Experimental Procedures". Fig 5.1,panel A shows the elution profile which occurs on DEAE-Sephadex column in the presence of Ca^{2+}-6M urea buffer. As can be seen, TnI, TnI-TnC complex, and TnC are well separated and the majority of the material remains as complex under these conditions. However, this CM-TnI-TnC complex could be dissociated into individual components by DEAE-Sephadex chromatography in the presence of 1 mM EGTA (Fig. 5.1, profile B). This result suggested that S-carboxamidomethylation does not affect the affinity of rabbit skeletal TnC for bovine cardiac CM-TnI.

Before cross-linking AGC-TnC to CM-TnI, it was necessary to verify that AGC-TnC still binds to CM-TnI. Perry(1979) showed that carboxamidomethylated TnC and the peptide CB9 both retained the following biological activities: to bind calcium with high affinity, to form a complex with TnI that is stable in high urea concentration and is calcium dependent, to neutralize the inhibition of Mg^{2+}-stimulated actomyosin ATPase produced by TnI and to inhibit phosphorylation of TnI from rabbit fast skeletal



Elution Profile	Proteins[a]	Treatment Condition Prior to Chromatography	Condition of Chromatography	Column Buffer Additives
A	CM-TnI:TnC Molar Ratio (1:1)	Complex Formation[b]	Complex Maintenance[c]	3mM Ca^{++} 1mM DTT
B	CM-TnI:TnC Molar Ratio (1:1)	Complex Formation	Complex Dissociation[d]	1mM EGTA 1mM DTT
C	CM-TnI:AGC-TnC Molar Ratio (1 : 1)	Complex Formation	Complex Dissociation	1mM EGTA No DTT
D	CM-TnI:AGC-TnC Molar Ratio (1 : 1)	1. Complex Formation 2. Photolysis[e]	Complex Dissociation	1mM EGTA No DTT
E	Isolated CM-TnI-AGC-TnC Complex (Panel D)	1. Complex Dissociation 2. Crosslinker Dissociation[f]	1. Complex Dissociation 2. Crosslinker Dissociation	1mM EGTA 1mM DTT

Elution Profile
CM-TnI
Complex
CM-TnI----TnC
TnC
.4
.3
.2
.1
28
20
12
4
Conductivity (mMHO)
1.2
.9
.6
.3
Absorbance 280nm (——)
4
2
0
Complex
CM-TnI-AGC-TnC
I
II
III
1.6
1.2
.8
.4
IV
V
VI
VII
VIII
Radioactivity X 10³ cpm per ml (–•–•–)
1
10
30
50
70
Fraction Number

Fig. 5.1.1 Chromatographic separation of TnI, TnC, and the TnI-TnC complex on DEAE A-50 Sephadex. See the text for the chromatographic conditions.

muscle by 3'-5'-cyclic AMP-dependent protein kinase. Head and Perry(1974) showed that the [^{14}C]-carbamoylmethylated TnC could be displaced by unmodified TnC from TnI suggesting a higher affinity of the untreated TnC for TnI. Similarly we have found that AGC-TnC binds to TnI only in the presence of Ca^{2+} using polyacrylamide gel electrophoresis(Tris-glycine buffer, pH8.6, and 6M urea, Fig. 5.2a). The AGC-TnC-(CM-TnI) complex though stable in 6M urea Tris-glycine polyacrylamide gel electrophoresis, was not stable to the conditions of DEAE-Sephadex chromatography even in the presence of calcium. This verifies the higher affinity of the untreated TnC for CM-TnI.

The stability of the AGC-moiety on TnC to the conditions of complex formation, complex dissociation and DEAE-Sephadex chromatography in 6M urea is shown in Fig. 5.1, profile C. All radioactivity remained attached to TnC and no transfer of the reagent to CM-TnI was observed. The AGC-moiety can be completely removed from AGC-TnC by reduction with DTT. These results suggested that the AGC-moiety is inert and that AGC-protein can be easily manipulated to test for the correct binding in the complex formation.

2. Isolation Of The Covalently Cross-linked CM-TnI-AGC-TnC Complex After Photolysis

In our control experiments(panel C of Fig. 5.1), the noncovalent complex between CM-TnI and AGC-TnC is completely

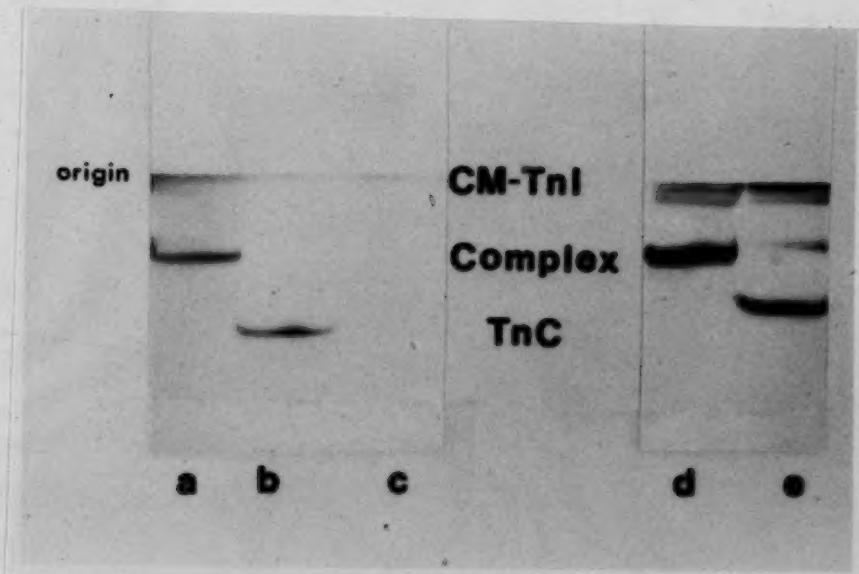


Fig. 5.2. Complex formation between bovine cardiac S-carboxamidomethylated troponin I (CM-TnI) and AGC-TnC. Polyacrylamide gels (7.5%) were polymerized in Tris/glycine buffer, pH 8.6, 6M urea. Samples (30-40 ug) were taken from stock solutions prepared as follows: AGC-TnC or untreated TnC dissolved in 0.1 ml of 50 mM Tris-HCl, 0.2M KCl buffer, pH 7.5, and CM-TnI dissolved in 0.1 ml of 50 mM HCl, 0.2M KCl were mixed in 1:1 protein molar ratio and 0.1 ml of either EGTA or $CaCl_2$ solutions were added to give final concentrations of 10 mM $CaCl_2$ and 10 mM EGTA. The solutions were allowed to stand for 1 hr at room temperature and then lyophilized, and the solid was redissolved in 0.2 ml of the Tris/glycine buffer, pH 8.6, containing 6M urea prior to electrophoresis. a, $+Ca^{2+}$, no photolysis, complex CM-TnI-AGC-TnC; b, $+Ca^{2+}$, no photolysis, AGC-TnC; $+Ca^{2+}$, no photolysis, CM-TnI(TnI stays at origin and is not seen in this gel.); d, EGTA, photolysis, complex CM-TnI-AGC-TnC (see peak II, panel D, Fig. 5.1); e, EGTA, photolysis, complex CM-TnI-AGC-TnC (see peak II, panel D, Fig. 5.1) treated with dithiothreitol.

dissociated by DEAE-Sephadex Chromatography in 6M urea and 1 mM EGTA. In contrast, after photolysis there is the formation of covalently cross-linked complex which can be separated by chromatography from the individual components (CM-TnI and AGC-TnC) (see panel D of Fig. 5.1). This covalently cross-linked complex was identified as CM-TnI-AGC-TnC based on the following results. Tris-glycine-urea polyacrylamide gel electrophoresis in the presence of EGTA, showed the cross-linked complex(Fig. 5.1, panel D, peak II) migrating as a complex formed from TnC and CM-TnI(Fig. 5.2d). Under the same conditions, the noncovalently linked CM-TnI-TnC complex will dissociate into its individual components. As mentioned before, this noncovalently linked complex exists only in the presence of calcium(Fig. 5.2a). Similarly, in the absence of reducing agents the covalently cross-linked product migrates with a molecular weight of approximately 45,000 daltons on the SDS-urea gel electrophoresis(Fig. 5.3g). On the other hand,in the presence of reducing agent this complex(peak II) behaved as noncovalently linked CM-TnI-TnC complex and dissociated into its components on the Tris-glycine-urea gel electrophoresis(Fig. 5.2e). Moreover, the radioactivity measurements in conjunction with amino acid analysis indicated that this cross-linked complex was a 1:1 complex of CM-TnI and AGC-TnC. The most convincing evidence comes from the isolation of CM-TnI-AGC from this cross-linked complex using DEAE-Sephadex chromatography in the presence of reducing agent, DTT(see below and Fig. 5.1, panel E, peak IV).

As identified by SDS-urea gel electrophoresis and amino acid analysis,peak I(Fig. 5.1, panel D) contained CM-TnI with no radioactivity indicating the absence of thiolysis between AGC and TnC during the photolysis of the reconstituted CM-TnI and AGC-TnC complex . This result agrees with our previous observation of the stability of the disulfide bond between AGC and the SH groups of proteins (Chapter IV). Also, peak III (Fig. 5.1, panel D) was identified using SDS-urea gel electrophoresis(Fig. 5.3f) as AGC-TnC, which did not cross-link to CM-TnI during the photolysis.

The aryl nitrene generated from the aryl azide of AGC-TnC during photolysis can react with either CM-TnI, TnC, or solvent (Fig. 5.4). If the aryl nitrene reacted with solvent, the only attachment of AGC to TnC was via the disulfide bridge. The addition of reducing agents such as DTT would remove the radiolabel marker from TnC. Radioactivity remaining on the covalently cross-linked complex and TnC (peak II and III) after the treatment with reducing agent would represent the amount of productive cross-linking of AGC to CM-TnI and intra-crosslinking of AGC to TnC, respectively. Dialysis of peaks II and III against solution of 2 mM β-mercaptoethanol was carried out. Radioactivity measurements and amino acid analyses indicated that 30%, 12%, and 58% of AGC-TnC had cross-linked to

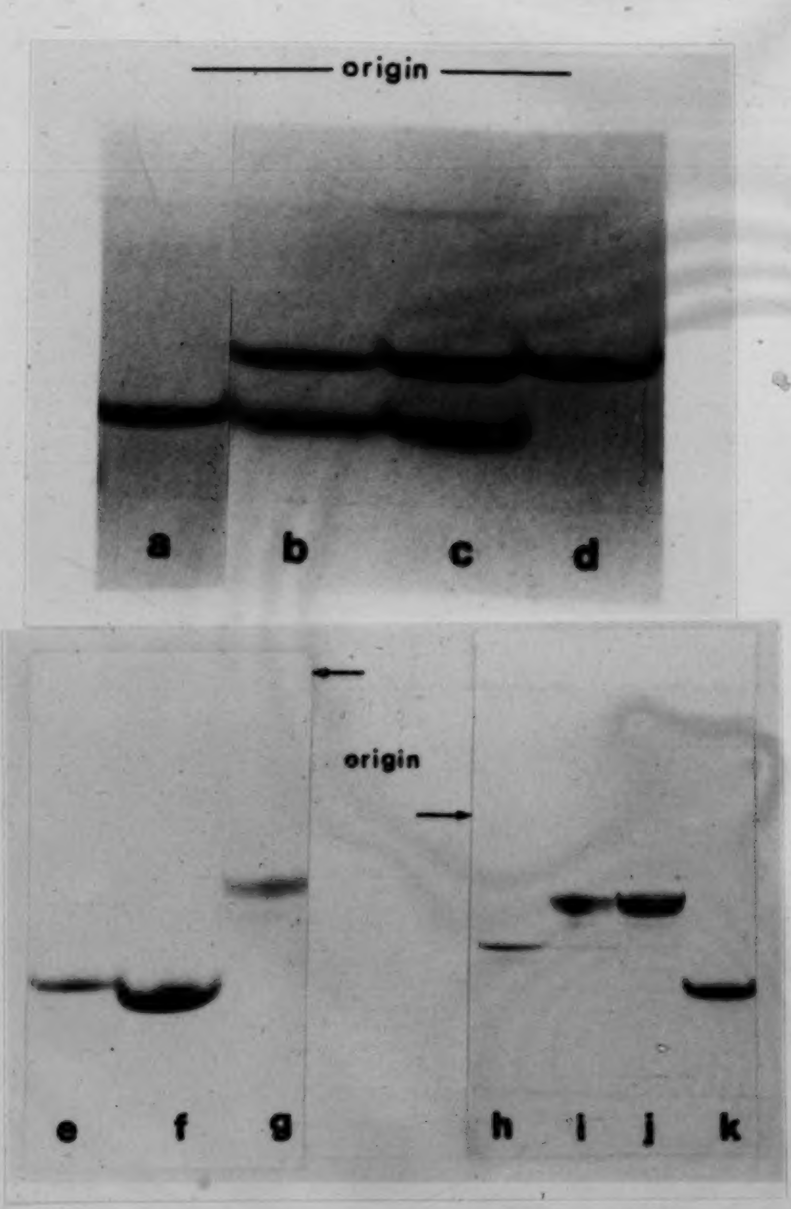


Fig. 5.3. SDS-urea polyacrylamide gel electrophoresis of CM-TnI, AGC-TnC and CM-TnI-AGC-TnC complex fractions from DEAE-chromatography. Samples a-d are prior to photolysis and chromatography; samples e-k are after photolysis and chromatography. a, AGC-TnC; b, complex AGC-TnC-CM-TnI; c, complex TnC-CM-TnI; d, CM-TnI; e, CM-TnI(see peak I, panel D, Fig. 5.1), no DTT; f, AGC-TnC(see peak III, panel D, Fig. 5.1), no DTT; g, complex CM-TnI-AGC-TnC (see peak II, panel D, Fig. 5.1), no DTT; h, CM-TnI-AGC (see peak IV, panel E, Fig. 5.1),+DTT; i, nonreversible cross-linked complex (see peak VI, panel E, Fig. 5.1), +DTT; j, nonreversible cross-linked complex (see peak VII, panel E, Fig.5.1), +DTT; k, TnC (see peak VIII, panel E, Fig. 5.1), +DTT.

CM-TnI, TnC itself, and solvent, respectively.

3. Isolation Of CM-TnI-AGC From The Covalently Cross-linked Complex

The reduction of the complex(Fig. 5.1, panel D, peak II) was shown on the Tris-glycine-urea gel electrophoresis to generate two additional bands corresponding to TnC and CM-TnI on the gel(Fig. 5.2e). Therefore, this covalently cross-linked complex was dissolved in 10 mM DTT, 10 mM EGTA, 8M urea, 50 mM Tris-HCl buffer, pH7.8 and the solution let stand at room temperature for 2 hr to ensure reduction of the disulfide bond between AGC and TnC. The solution was then dialyzed against the DEAE-Sephadex column starting buffer. The sample was applied to the DEAE-Sephadex column and the elution profile was shown in Fig. 5.1, panel E. Four protein peaks denoted IV, VI, VII, and VIII were collected and each peak dialyzed against 1 mM HCl, 2 mM β-mercaptoethanol and lyophilized. Peak V is not protein as indicated from lack of absorption at 230 nm and amino acid analysis. The position of this peak corresonds to that of the reagent or photolyzed reagent, AGTC, run on the DEAE-Sephadex column. The only difference in treatment of the proteins between panel D and E of Fig. 5.1 is the addition of DTT. This photolysis by-product may be tightly bound to CM-TnI or TnC such that it is not removed by dialysis prior to chromatography but is separated from its ligand by ion-exchange on the DEAE-Sephadex column. The

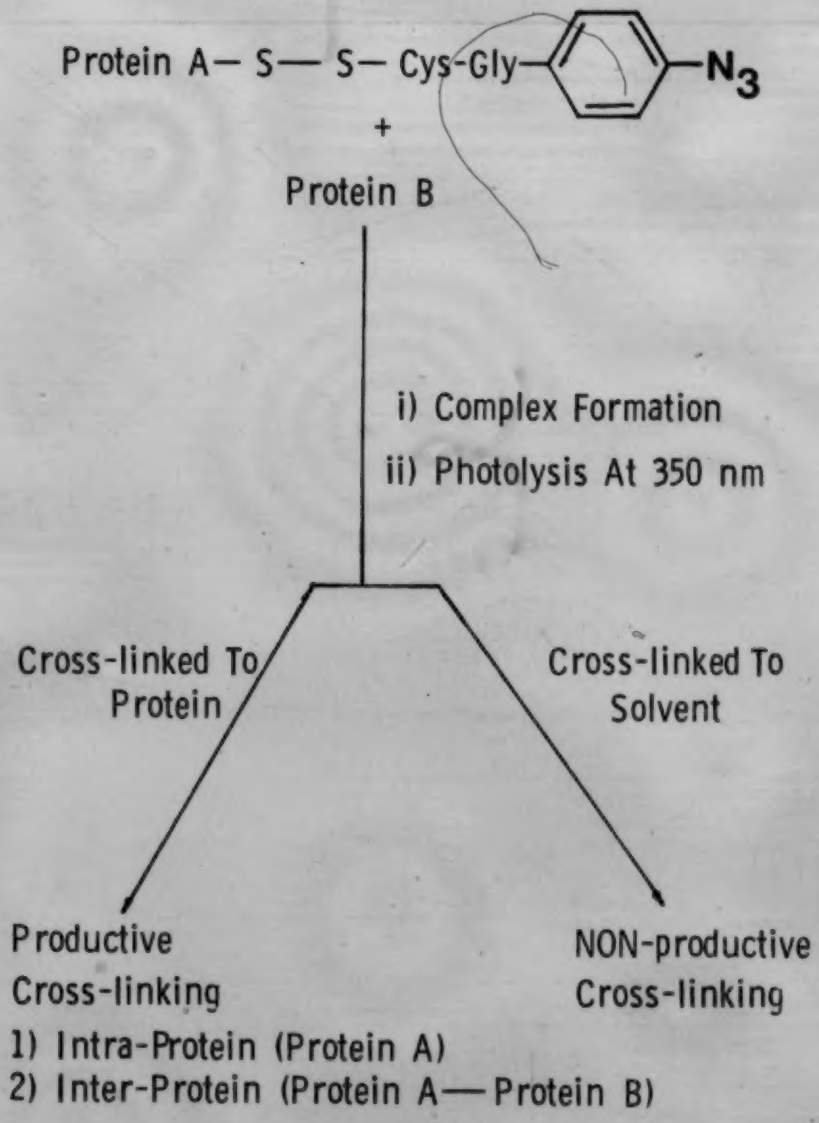

Protein A— S— S— Cys-Gly—
N3
+
Protein B
i) Complex Formation
ii) Photolysis At 350 nm
Cross-linked To Protein
Cross-linked To Solvent
Productive Cross-linking
NON-productive Cross-linking
1) Intra-Protein (Protein A)
2) Inter-Protein (Protein A—Protein B)

Fig. 5.4 The fate of Aryl Nitrene generated from the aryl azide of AGC-Protein.

expected product of the reduction of the covalently cross-linked complex was CM-TnI-AGC as identified on SDS-urea gel electrophoresis (Fig. 5.3h). Using radioactivity measurements and amino acid analysis, peak IV was shown to contain 54% of the radiolabel.

Two additional radioative protein peaks were obtained, peak VI and peak VII representing 20% and 26% respectively of the radiolabelled proteins. Peak VII which eluted in the position of CM-TnI-AGC-TnC complex (peak II, panel D of Fig. 5.1) was retreated with DTT as described above and rerun on DEAE-Sephadex column. This fraction eluted as a single radioactive peak in the identical position. SDS-urea gel electrophoresis in the presence of β-mercaptoethanol showed that this fraction migrated to the identical position of the complex with no appearance of CM-TnI and TnC(Fig. 5.3j). Similarly, peak VI comigrated on SDS-urea gel electrophoresis to the position of the complex (Fig. 5.3i). Therefore, these protein fractions represent non-reversible complex and no further studies have been carried out. The other expected product of the reduction of the covalently cross-linked complex is TnC (peak VIII, panel E of Fig. 5.1) as identified on SDS-urea gel electrophoresis (Fig. 5.3k). This TnC as expected contained no radioactivity indicating the complete removal of radioactive AGC by reduction.

D. Discussion

In this Chapter I have described the use of DEAE-Sephadex chromatography as a method to identify and purify TnI-TnC complex. We have shown that a 1:1 complex can be formed between bovine cardiac CM-TnI and rabbit skeletal TnC. This complex is stable to DEAE-Sephadex chromatography in 6M urea but can be dissociated in the SDS-urea polyacrylamide gel electrophoresis. Moreover, the formation of the complex is calcium dependent and can be dissociated by DEAE-Sephadex chromatography in the presence of 6M urea and 1 mM EGTA.

Cysteine 98 of rabbit skeletal TnC can be quantitatively modified with AGTC. The AGC-TnC forms a complex with rabbit skeletal CM-TnI and this complex is stable in the presence of calcium to Tris-glycine-urea polyacrylamide gel electrophoresis but not to the conditions of DEAE-Sephadex chromatography. These results suggest that though the interactions between CM-TnI and AGC-TnC in the complex are strong, the affinity of unmodified TnC is higher for CM-TnI. These results also support the conclusion that cysteine 98 of TnC is in close proximity of the site of interaction between TnI and TnC.

The reason for using bovine cardaic CM-TnI to form a complex with rabbit skeletal TnC was that rabbit skeletal CM-TnI would not form a complex that was stable to the conditions of DEAE-Sephadex chromatography. Comparison of the amino acid sequences of rabbit cardiac TnI (containing 2

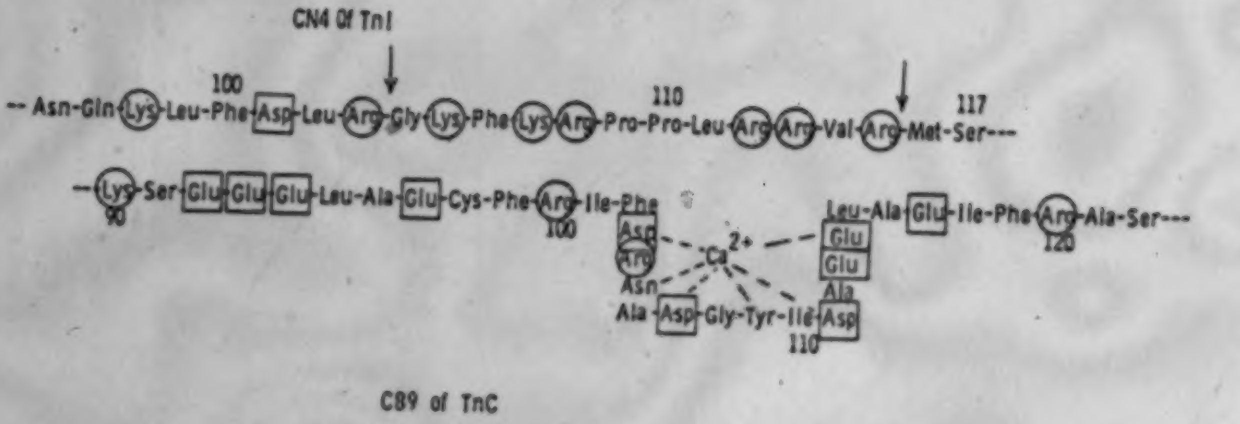

CN4 Of TnI
-- Asn-Gln-Lys-Leu-Phe-Asp-Leu-Arg-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-Met-Ser---
100
110
117
--Lys-Ser-Glu-Glu-Glu-Leu-Ala-Glu-Cys-Phe-Arg-Ile-Phe
90
100
Asp
Arg
Asn
Ala-Asp-Gly-Tyr-Ile-Asp
110
Ca2+
Ala
Glu
Glu
Leu-Ala-Glu-Ile-Phe-Arg-Ala-Ser---
120
CB9 of TnC

Fig. 5.5 Regions of the polypeptide chains of rabbit skeletal troponin I and troponin C involved in interaction. [From Perry, 1979]

cysteine residues) and rabbit skeletal TnI(containing 3 cysteine residues) shows that both proteins have cysteine residues in homologous positions 48 and 64 using the rabbit skeletal sequence whereas rabbit skeletal TnI has a third cysteine residue at position 133. One might expect the bovine cardiac TnI to be largely homologous to that of rabbit cardiac TnI since the sequences of TnI from different sources appear to be tissue specific and not as homologous when compared within a species of different tissues(Grand and Wilkinson, 1977). Therefore, the above results suggest that S-carboxamidomethylation of cysteine 133 of rabbit skeletal TnI accounts for the lack of complex formation with TnC on DEAE chromatography and that cysteine 133 is in close proximity of the site of interaction between TnI and TnC. It is also consistent with the observation from the S-carboxamidomethylation of TnI that cysteine 133 was partially protected by TnC in the presence of calcium (Chapter III). Interestingly, cysteine 133 is the closest cysteine residue in the linear sequence to serine 117 and to the site on TnI (residues 105-114) which inhibits the actomyosin ATPase(Talbot and Hodges, 1981 a and b). The other reason for bovine cardiac CM-TnI having higher affinity for TnC than rabbit skeletal CM-TnI could simply be that bovine cardiac TnI has a different structure at that particular binding region.

Furthermore, Perry(1979) has postulated that the interaction between TnC and TnI probably has considerable electrostatic character. The phoshorylation sites on TnI that are blocked on interaction with TnC are located in regions that have an excess of basic amino acid residues, and the peptide CB9 of TnC has an excess of aspartic and glutamic acid residues(Fig. 5.5). Calcium strengthens the interaction of TnI with TnC and is essential for the interaction of CB9 with TnI. This suggests that the acidic residues involved in the binding of calcium may not be directly implicated in the interaction between the two proteins. If the binding site of TnI for CB9 of TnC was around the cysteine 133 region, then a likely explanation would be that the conformational changes induced by calcium would bring the adjacent negatively charged residues(not involved in cation binding) into a more favorable position for interaction with the appropriate residues on TnI, which may have different conformation when cysteine 133 is modified (see Fig 5.5).

Photolysis of the reconstituted binary complex of bovine cardiac CM-TnI and AGC-TnC resulted in the formation of a covalently cross linked 1:1 CM-TnI-AGC-TnC complex in 30% yield. This cross-linked complex could be isolated from the photolyzate by DEAE-Sephadex chromatography in 6M urea and 1 mM EGTA in the absence of reducing agents. The isolated cross-linked complex could be treated with DTT to reduce the disulfide bond between AGC and TnC to complete the transfer of the radiolabel AGC from cysteine 98 of TnC to CM-TnI. The CM-TnI-AGC was isolated from TnC by DEAE chromatography in the presence of reducing agent, DTT, in a 54% yield. The formation of the covalent bond between the aryl nitrene and TnI indicates that we have successfully demonstrated and radiolabelled a region on TnI within 14 Å distance of cysteine 98 of TnC. Sequence analysis of this radiolabel CM-TnI is required to identify the amino acid residues on TnI which are at the site of interaction.

VI. THE REGION ON TROPONIN IN CLOSE PROXIMITY TO CYSTEINE 190 OF TROPOMYOSIN

A. Tropomyosin-Troponin Interaction

In Chapter I we have discussed the evidence suggesting that the site of Tn-binding is about 1/3 of the distance from the COOH-terminal end of TM. Also, the fragment studies indicated that two regions of TnT may be involved in the binding of the troponin complex to tropomyosin. It can be argued that the interaction properties of fragments of TnT and even TnT may not necessarily reflect those of the native troponin molecule. Therefore the present study was carried out to provide direct evidence for the region of troponin that is in close proximity to Cys-190 of TM in the presence and absence of Ca^{2+}. Our approach was to selectively modify cysteine 190 of TM with the heterobifunctional photoaffinity probe, N-(4-azidobenzoyl-[2-^{3}H]-glycyl)-S-(2-thiopyridyl)-cysteine (AGTC) via disulfide bond formation, followed by covalent crosslinking of the probe to troponin by photolysis. Reduction of the disulfide bond between TM and the photoaffinity probe, isolation of radiolabelled troponin followed by chymotryptic digestion enabled the identification of the radiolabelled region. This strategy for the use of AGTC has been previously described in Chapter IV and successfully demonstrated with the TnI-TnC interaction in Chapter V.

B. Experimental Procedures

1. Preparation of AGC-Tropomyosin

The introduction of the photoaffinity probe (arylazide structure) into rabbit cardiac α-tropomyosin at cysteine 190 has been described in Chapter IV with the following modification. α-Tropomyosin (132 mg) was dissolved in 10.0 ml of 8 M urea, 50 mM Tris-HCl, 10 mM dithiothreitol, 1 mM EGTA buffer, pH 8.0, and dialyzed at 4°C overnight against 1 liter of 10 mM Tris-HCl, 0.1 M KCl, 1 mM dithiothreitol buffer, pH 7.5 with nitrogen gas being bubbled through the dialysate. This step ensured complete reduction of the free sulfhydryl group. Dithiothreitol was removed from the protein by gel filtration on a Sephadex G-25 column (1.5 x 100 cm) eluted with 10 mM Tris-HCl, 0.1 M KCl buffer, pH 7.5. To the protein solution (35 ml) was added 70 mg of radioactive solid AGTC, so that the molar ratio of sulfhydryl content in TM to the photoaffinity probe was 1:75. The reaction mixture was adjusted to pH 8.0 with a 2 M Tris solution and stirred in the dark at room temperature for 6 hr, and then the solution was lyophilized. The lyophilized material was dissolved into 10 ml of distilled water and stirred for 2 hr. The excess AGTC was removed by gel filtration on a Sephadex G-25 column (1.5 x 100 cm) previously equilibrated with 10 mM Tris-HCl, 0.1 M KCl buffer, pH 7.5. The incorporation of the reagent was determined by radioactivity measurements and amino acid analysis. Usually, 90-100% modification was achieved.

2. Conditions For Noncovalent Complex Formation Between Tropomyosin and S-carboxamidomethylated Native Troponin

In complex formation experiments between α-tropomyosin and native troponin, α-tropomysoin and S-carboxamidomethylated native troponin, and AGC-tropomyosin and S-carboxamidomethylated native troponin, the following method was used. CM-Tn (70 mg, 1 umole) was dissolved in 10 ml of 10 mM Tris-HCl, 0.1 M KCl, 1 mM dithiothreitol buffer, pH 7.5, containing either 1 mM EGTA or 3 mM $CaCl_2$, and then dialyzed at 4°C for 4 hr against 1 litre of the same buffer. Dithiothreitol was removed by gel filtration on a Sephadex G-25 column (1.5 x 60 cm) which was previously equilibrated with the above buffer without dithiothreitol. To this freshly reduced troponin (25 ml) a solution of AGC-TM (0.67 umole/30 ml) in 10 mM Tris-HCl, 0.1 M KCl buffer, pH 7.5, was added dropwise over a 5 min period. The protein mixture was stirred under nitrogen at 4°C for 30 min, transferred into the photolysis reaction vessel and saturated with nitrogen for a further 30 min before the photolysis.

3. Isolation of Covalently Linked CM-Tn-AGC-TM

Isolation of covalently linked CM-Tn-AGC-TM complex after photolysis was carried out using hydroxylapatite chromatography as described in Chapter II.

4. Isolation Of CM-Tn-AGC From CM-Tn-AGC-TM Complex

Tropomyosin and the covalently linked CM-Tn-AGC-TM complex isolated from the method described above were dissolved in 8 ml of 8 M urea, 50 mM Tris-HCl, 1 M KCl, 10 mM dithiothreitol buffer, pH 8.0, and stirred at 65°C for 15 min, then 2 hr at room temperature. This step ensured complete reduction of the disulfide bond between tropomyosin and the crosslinker. The protein solution was dialyzed at 4°C for 4 hr against 1 litre of the starting buffer, 10 mM Na_2HPO_4, 1 M KCl, 2 mM β-mercaptoethanol, pH 7.0, and applied to a hydroxylapatite column (1.0 x 40 cm). The column was eluted as described above except that all buffers contained 2 mM β-mercaptoethanol. The effluent was monitored both by radioactivity measurements and absorbancy at 230 nm. The protein fractions were dialyzed against 2 mM NH_4HCO_3 containing 2 mM β-mercaptoethanol and lyophilized.

5. Limited Proteolysis Of Troponin With TLCK-Chymotrypsin

The following method was used for the proteolytic cleavage of native troponin, CM-Tn and CM-Tn-AGC with TLCK-chymotrypsin. CM-Tn (30 mg) was dissolved in 9 ml of 50 mM Tris-HCl, 0.1 M NaCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 1 mM dithiothreitol buffer, pH 8.0. The protein solution was stirred at 4°C for 1 hr. TLCK-chymotrypsin (0.3 mg) was added directly into the CM-Tn solution, so that the enzyme to CM-Tn ratio was 1:100 by weight. The reaction mixture was stirred for 15 min at 4°C. The reaction was stopped by the addition of 10 ul of 1 M diisopropyl phosphofluoridate (DFP). After another 10 min of stirring, the protein solution was freeze-dried and dialyzed against the starting buffer for the DEAE-Sephadex A-50 chromatography. The digest was separated on this column using the conditions described above.

6. Purification Of T2-AGC On TM-Sepharose 4B Affinity Chromatography

The chromatographic conditions used for the separation of T2 and T2' are those described by Pearlstone and Smillie (1981).

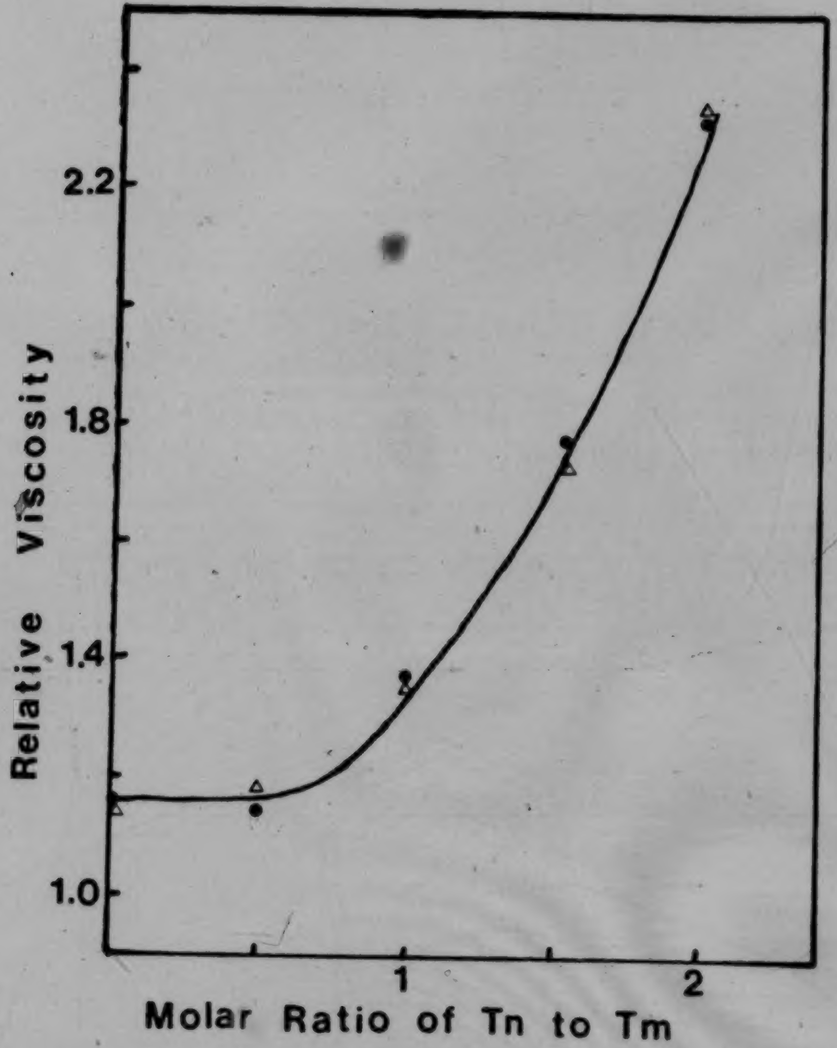


FIG. 6.1. Effect of the addition of native troponin (Δ-Δ-Δ) and the S-carboxamidomethylated troponin (●-●-●) on the relative viscosity of α-tropomyosin; α-tropomyosin was 0.5 mg/ml in 10 mM Tris-HCl, 0.1 M KCl buffer, pH 7.5.

C. Results And Discussion

1. Effect of S-carboxamidomethylation of Tn on Its Function

In Chapter III, we found that cysteine 133 of TnI in native rabbit skeletal troponin was accessible to alkylation with iodoacetamide in the presence and absence of Ca^{2+}. In order to eliminate the possibility of disulfide interchange between native troponin and AGC-TM, this exposed -SH group was S-carboxamidomethylated with iodoacetamide. The effect of this chemical modification on native troponin was tested by the ability of CM-Tn to interact with TM. Under the conditions described in the "Experimental Procedures", CM-Tn was able to form a noncovalent complex with TM and to promote the head-to-tail polymerization of TM as shown by the increase in viscosity (Fig. 6.1). This result is consistent with the results of Ohyashiki and Sekine(1979) who found that after modification of Tn with either of the two SH-directed fluorogenic reagents, N-(p-(2-benzimidazolyl)phenyl)maleimide and N-(1-anilinonaphthyl-4) maleimide, the Tn was able to interact with TM in the presence and absence of Ca^{2+}. Furthermore, CM-Tn still maintains its Ca^{2+} sensitizing activity in the actomyosin ATPase assay. Also, Sutoh and Matsuzuki(1980) found that the Tn reconstituted from TnT, TnC and alkylated TnI had the same calcium sensitivity as native Tn on the actomyosin ATPase. These results indicate that S-carboxamidomethylation does not affect the biological integrity of Tn.

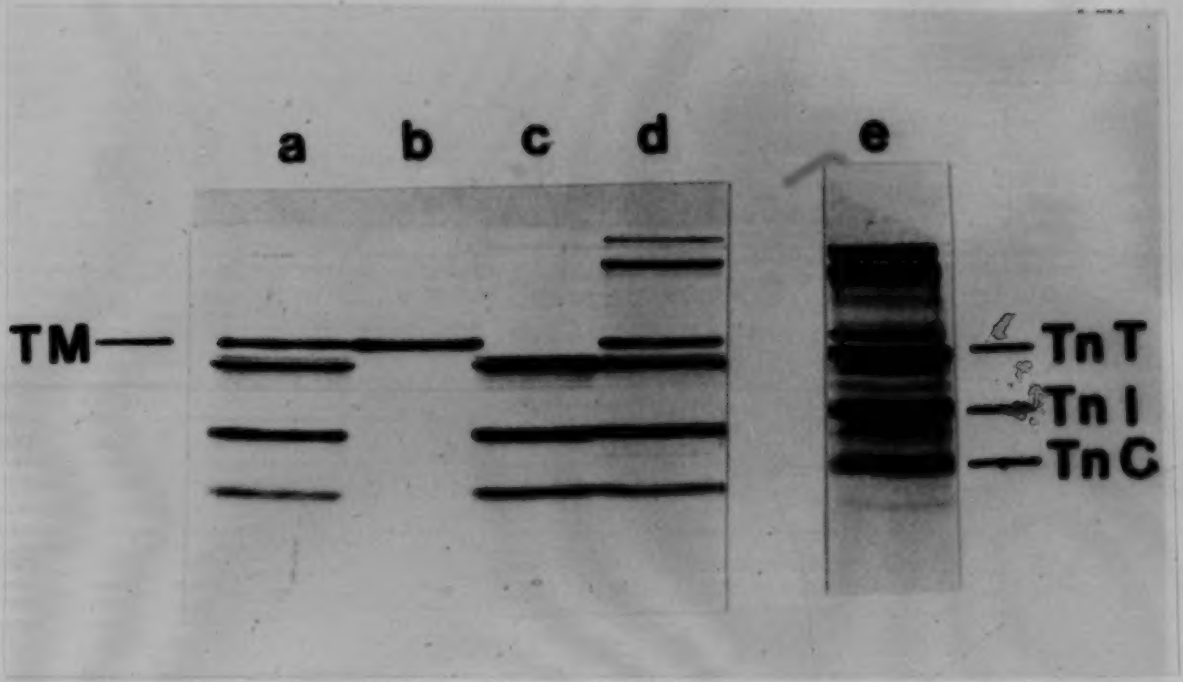


FIG. 6.2. Sodium dodecyl sulfate-urea Laemmli gel electrophoresis of the complex formed between CM-troponin and either tropomyosin or AGC-tropomyosin after photolysis. a, Photolyzed complex of CM-Tn and TM, + β-mercaptoethanol; b, AGC-TM before photolysis; c, CM-Tn before photolysis, + β-mercaptoethanol; d, Photolyzed complex of CM-Tn and AGC-TM, + β-mercaptoethanol; e, Photolyzed complex of CM-Tn and AGC-TM, without β-mercaptoethanol.

2. Photochemical Cross-linking of The Reconstituted Regulatory Complex

The reconstituted complex of CM-Tn and AGC-TM was irradiated at 4°C for 4 hr in 10 mM Tris-HCl, 0.1 M KCl buffer, pH 7.0 in the presence of 3 mM $CaCl_2$ or 1 mM EGTA at a wavelength of 350 nm to form the covalently crosslinked complex between cysteine 190 of TM and Tn. The crosslinked products were similar in both conditions as shown by Laemmli gel electrophoresis (Fig. 6.2e). The apparent molecular weights of the crosslinked products as estimated from their mobilities in the gel electrophoresis were approximately 64,000 and 100,000 daltons. These molecular weights correspond to the complexes TnT-TM (63,000), TM-dimer (66,000), and the complex of TM-TnT-TM (100,000). In the presence of β-mercaptoethanol the disulfide bond between the crosslinker and TM should be reduced and no complexes should be observed on gel electrophoresis. However, some irreversible cross-linked product was seen on gel electrophoresis (Fig. 6.2d) At this time, no definitive explanation can be given for such irreversible crosslinked complexes. It must be noted that no crosslinking was observed when CM-Tn mixed with unmodified TM was irradiated (Fig. 6.2a).

3. Isolation of Covalently linked CM-Tn-AGC-TM complex after Photolysis

Eisenberg and Kielley(1974) have shown that TM can be separated from Tn by hydroxylapatite column chromatography. Therefore after photolysis the reaction products were separated on hydroxylapatite chromatography in the absence of reducing agent (Fig. 6.3) as described in the "experimental procedures". Noncomplexed CM-Tn was obtained in Peak I, Fig. 6.3, and a mixture of CM-Tn-AGC-Tm and AGC-TM in peak II, Fig. 6.3. Greater than 90% of the radioactivity was observed in peak II as expected. An attempt to separate the CM-Tn-AGC-TM complex from AGC-TM on the hydroxylapatite column with a phosphate gradient was unsuccessful.

In our control experiment the noncovalent CM-Tn-TM complex is dissociated into TM,TnT, TnI and TnC on SDS-urea Laemmli gel electrophoresis (Fig. 6.2a). However, in the absence of reducing agent, Peak II, Fig. 6.3 was dissociated into TM, TnI, TnC and high molecular weight components on Laemmli gel electrophoresis (Fig. 6.3, insert). In the presence of excess β-mercaptoethanol, an additional band corresponding to TnT was observed on the gel (Fig. 6.3, insert). These results suggested that TnT is in close proximity to cysteine 190 of TM (within 14 Å) in the presence and absence of Ca^{2+}.

The arylnitrene generated from AGC-TM during photolysis could react with either CM-Tn, solvent, or TM(Fig. 5.4). When the arylnitrene reacts with solvent the only attachment of AGC to TM is via the disulfide bond. In this case the

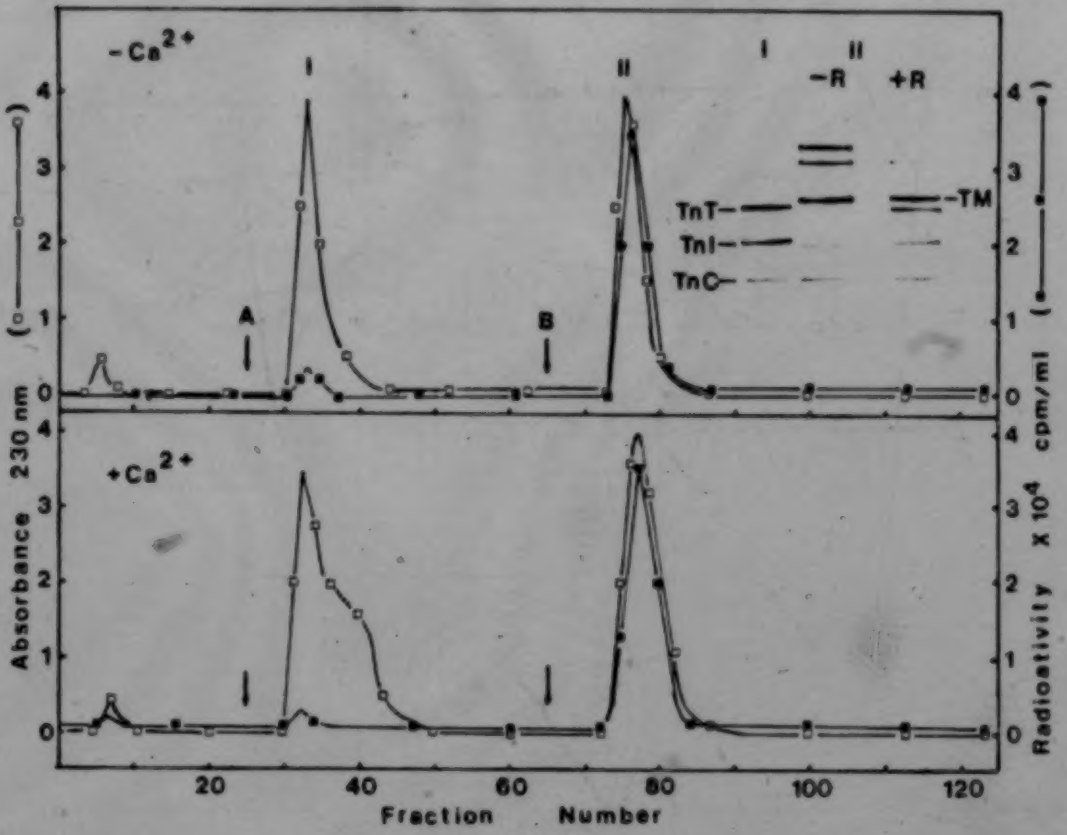


FIG. 6.3. Chromatographic separation of photolyzed complex of CM-troponin and AGC-tropomyosin on hydroxylapatite in the absence of reducing agent. $+Ca^{2+}$ or $-Ca^{2+}$ refers to the presence or absence of calcium in the buffer during photolysis. The column (1.0 x 40 cm) was equilibrated with 10 mM Na_2HPO_4, 1 M KCl buffer, pH 7.0. A and B indicate the start of 60 mM Na_2HPO_4, 1 M KCl buffer, pH 7.0, and 300 mM Na_2HPO_4, 1 M KCl buffer, pH 7.0 for column elution, respectively. The column effluent was monitored for absorbance at 230 nm (□-□) and radioactivity (■-■). Inset: SDS-urea Laemmli gel of Peaks I and II (+R and -R refer to the presence and absence of added β-mercaptoethanol in the sample for gel electrophoresis).

addition of reducing agent would remove the radiolabelled crosslinker. Reduction of Peak II in denaturant (8 M urea) and reducing agent was carried out to remove the crosslinker that had reacted with solvent (54% and 73% in the absence and presence of Ca^{2+}, respectively, Table 6-I). The radioactivity remaining in the protein fraction after exhaustive dialysis against 10 mM phosphate, 1 M KCl, 2 mM β-mercaptoethanol buffer, pH 7.0, would represent the amount of protein cross-linking (46% and 27% in the absence and presence of Ca^{2+}, respectively; Table 6-I). Radioactivity measurements indicated that the protein crosslinking yield increased 1.7 fold in the absence of calcium compared to that in the presence of calcium.

4. Isolation of CM-Tn-AGC from the Covalently Linked CM-Tn-AGC-TM Complex

The protein fraction (Peak II, Fig. 6.3) containing the covalent complex between CM-Tn and AGC-TM was dissolved in a denaturing medium containing reducing agent (8 M urea, 50 mM Tris-HCl, 1 M KCl, 10 mM dithiothreitol buffer, pH 8.0) to cleave the disulfide bond in the covalently linked CM-Tn-AGC-TM complex and generate the radiolabelled CM-Tn-AGC and TM. The CM-Tn-AGC was then isolated from TM and any TM-AGC by hydroxylapatite chromatography as described in the "Experimental Procedures". The CM-Tn-AGC, like native Tn, was eluted from the column with 60 mM phosphate buffer (Peak III, Fig. 6.4). SDS-urea Laemmli gels

TABLE 6-I

Photochemical Cross-linking Results of the Complex Formed Between CM-Tn AGC-TM in the Presence and Absence of Calcium

	Protein Fraction or Solvent	Experiment Number	Cross-linking Yield 1 mM EGTA[a] (%)		3 mM $CaCl_2$[a] (%)	
A	Total protein fraction[b] (CM-Tn-AGC and TM-AGC)	1	50.2	46.1	28.5	27.0
		2	42.0		25.5	
B	Solvent[c]	1	49.8	53.9	71.5	73.0
		2	58.0		74.5	
C	CM-Tn-AGC fraction[d]	1	24.7	24.0	25.5	24.9
		2	23.3		24.2	
D	TM-AGC[e]	1	75.3	76.0	74.5	75.1
		2	76.7		75.8	
E	CM-Tn-AGC[f] fraction (Overall yield)	1	12.4	11.1	7.3	6.8
		2	9.8		6.2	
F	TM-AGC[g] (Overall yield)	1	37.8	35.0	21.2	20.3
		2	32.2		19.3	

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[a] The complex was photolyzed in 0.1M KCl, 10 mM Tris-HCl buffer, pH7.5, containing either 1 mM EGTA or 3 mM $CaCl_2$ for 4 hr at 4°C at a wavelength of 350 nm.

[b] The ratio of radioactivity in the total protein fraction (CM-Tn-AGC and TM-AGC) after treatment with reducing agent over the total radioactivity in this fraction prior to treatment with reducing agent, time 100.

[c] The % loss in radioactivity on treatment with reducing agent represents amount of arylnitrene that reacted with solvent (B = 100 - A).

[d] The ratio of radioactivity found in the CM-Tn-AGC isolated by hydroxylapatite chromatography in the presence of reducing agent to the total radioactivity found in CM-Tn-AGC and TM-AGC on the same column, time 100.

[e] The ratio radioactivity found in TM-AGC isolated by hydroxylapatite chromatography in the presence of reducing agent to the total radioactivity found in CM-Tn-AGC and TM-AGC on the same column, time 100.

[f] Ratio of cross-linking to CM-Tn to the total cross-linking to CM-Tn, TM and solvent (E = A x C/100).

[g] Ratio of cross-linking to TM to the total cross-linking to CM-Tn, TM and solvent (F = A x D/100).

showed that Peak III contained all three components of Tn, that is, TnT, TnI and TnC (Fig. 6.4, insert). The additional radioactive protein peak(Peak IV, Fig. 6.4) was TM-AGC and irreversible high molecular weight complex as shown on the SDS-urea gel electrophoresis (Fig. 6.4, insert). At this time, no definitive explanation can be given for the appearance of irreversible crosslinked complexes. Since our interest is in the interaction between Tn and TM no further studies were carried out on Peak IV. Radioactivity measurements of peaks III and IV indicated that of the protein crosslinking yield 24% and 76% of the arylnitrene had crosslinked to CM-Tn and TM respectively in the absence of Ca^{2+} (Table 6-I). Similarly, in the presence of Ca^{2+}, 25% and 75% of the arylnitrene crosslinked to CM-Tn and TM respectively (Table 6-I). The overall crosslinking yield to Tn was 7% and 11% in the presence and absence of Ca^{2+} respectively (Table 6-I). In comparison the crosslinking to TM itself was 20% and 35% in the presence and absence of calcium (Table 6-I). These results indicated that the overall crosslinking yield to both Tn and TM increased approximately 1.7 fold in the absence of calcium compared to the presence of calcium. They suggest that there is a conformational change induced by calcium in the Tn-TM complex around cysteine 190 and that in the absence of calcium the complex is more compact or the binding is stronger around cysteine 190 such that there is less crosslinking of the arylnitrene to solvent.

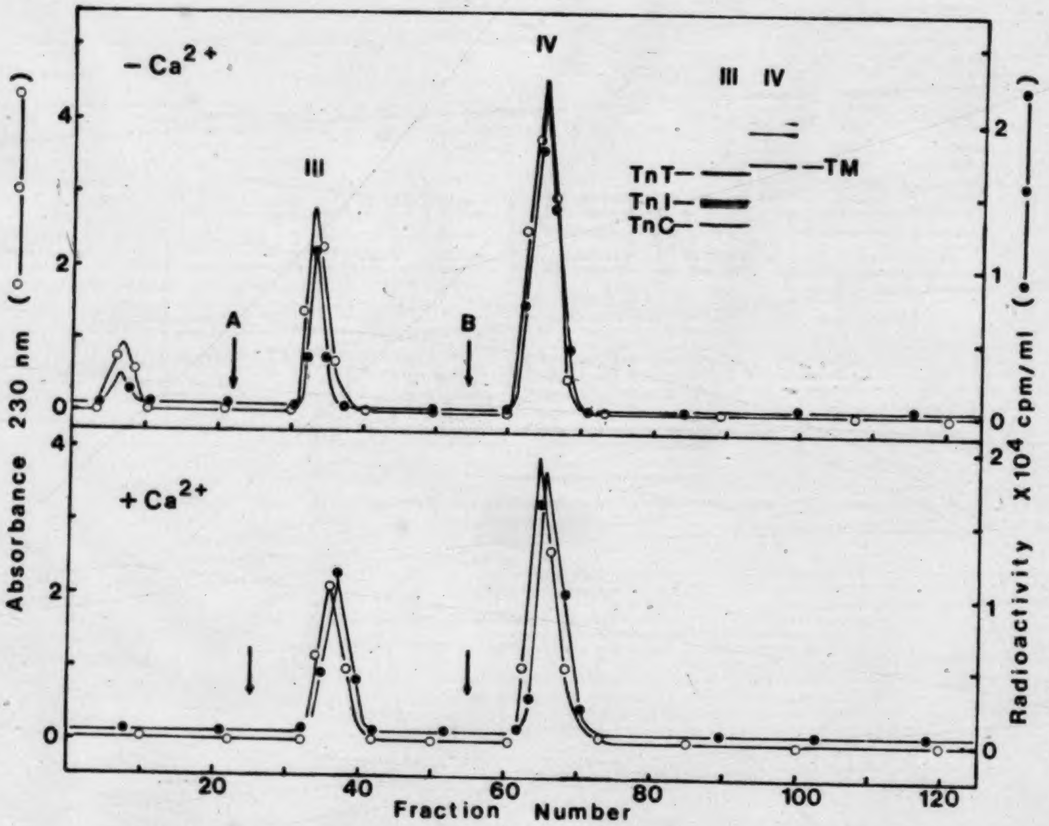

FIG. 6.4. Chromatographic separation of Peak II on hydroxylapatite in the presence of β-mercaptoethanol. Peak II was obtained from hydroxylapatite chromatography in the absence of reducing agent (Fig. 6.3) and contains the CM-Tn-AGC-TM covalent complex and modified tropomyosin. $+Ca^{2+}$ or $-Ca^{2+}$ refers to the presence or absence of calcium in the buffer during photolysis. The column (1.0 x 40 cm) was equilibrated with 10 mM Na_2HPO_4, 1 M KCl, 2 mM β-mercaptoethanol buffer, pH 7.0. A and B indicate the start of the same buffer except the phosphate concentration was incrased to 60 mM and 300 mM respectively. The column effluent was monitored for absorbancy at 230 nm (○-○) and radioactivity (●-●). Inset: SDS-urea Laemmli gel of Peaks III and IV in the presence of β-mercaptoethanol in the samples.

5. The Subunit of the Tn complex in the Vicinity of Cysteine 190 of TM

Previous studies (Potter and Gergely,1974; Maruyama et al,1975; Dahowska et al,,1976) have reported the possibility of interaction between TnI and TM. Also, the immunoelectron microscopic observation by Ohtsuki(1979) showed that the position of the individual antibodies against TnI and TnC on the thin filament were the same as the position of antibody against fragment T2 of TnT, that is, one-third the distance from the COOH-terminus of TM in the vicinity of cysteine 190 of TM. These results suggest that all three subunits of Tn may be in proximity to cysteine 190 and accessible to the photoaffinity probe AGTC attached to cysteine 190.

To determine which subunit of CM-Tn-AGC contained the radiolabel the CM-Tn-AGC complex(Peak III, Fig. 6.4) was separated into the individual subunits on DEAE-Sephadex A-50 chromatography as described in the "Experimental Procedures". Only Peak VI of Fig. 6.5 contained radioactivity and was shown to be TnT on SDS-urea Laemmli gel electrophoresis. Peaks V and VII were TnI and TnC respectively and contained no radioactivity. These results suggest TnT is in close proximity (within 14 Å) to cysteine 190 of TM in the presence and absence of Ca^{2+} while TnC and TnI are not. This result is in agreement with those of Sutoh and Matsuzuki(1980) who found no crosslinking between modified TnI and TM in the reconstituted thin-filament.

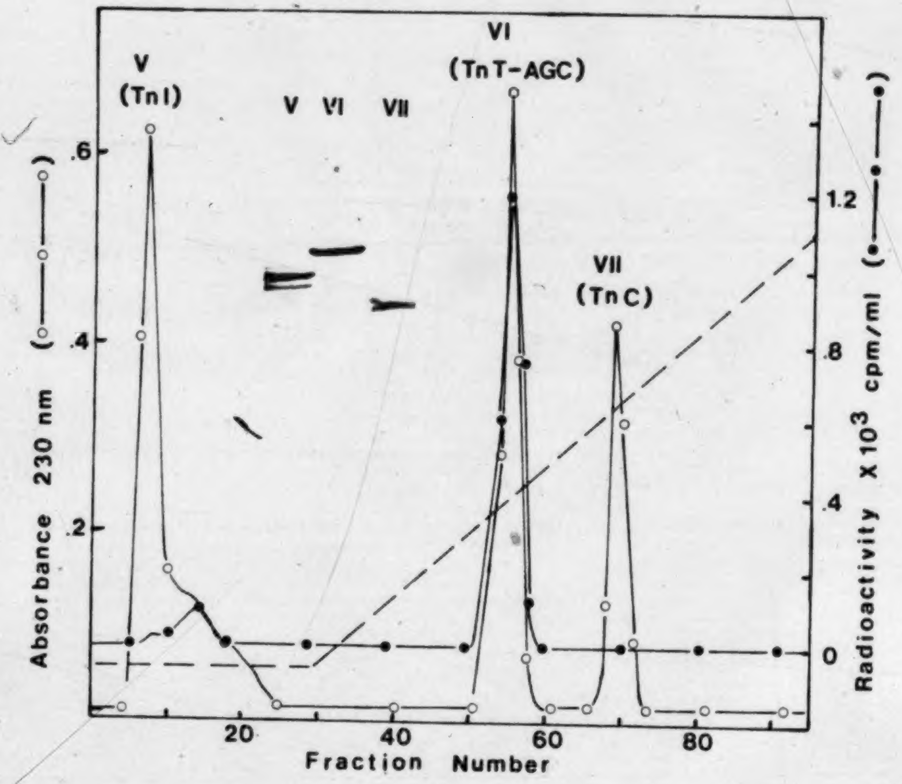

FIG. 6.5. Chromatographic separation of CM-Tn-AGC (Peak III, Fig. 4) on DEAE-Sephadex A-50. The solumn (1.6 x 40 cm) equilibrated with 50 mM Tris-HCl, 8 M urea, 1 mM dithiothreitol buffer, pH 7.8, was eluted with a linear salt gradient (-----) from 0 to 0.6 M KCl in the same buffer (150 ml each). The column effluent was monitored for absorbancy at 230 nm (○-○) and radioactivity (●-●). Inset: SDS-urea Laemmli gel of Peaks V, VI and VII in the presence of β-mercaptoethanol in the samples.

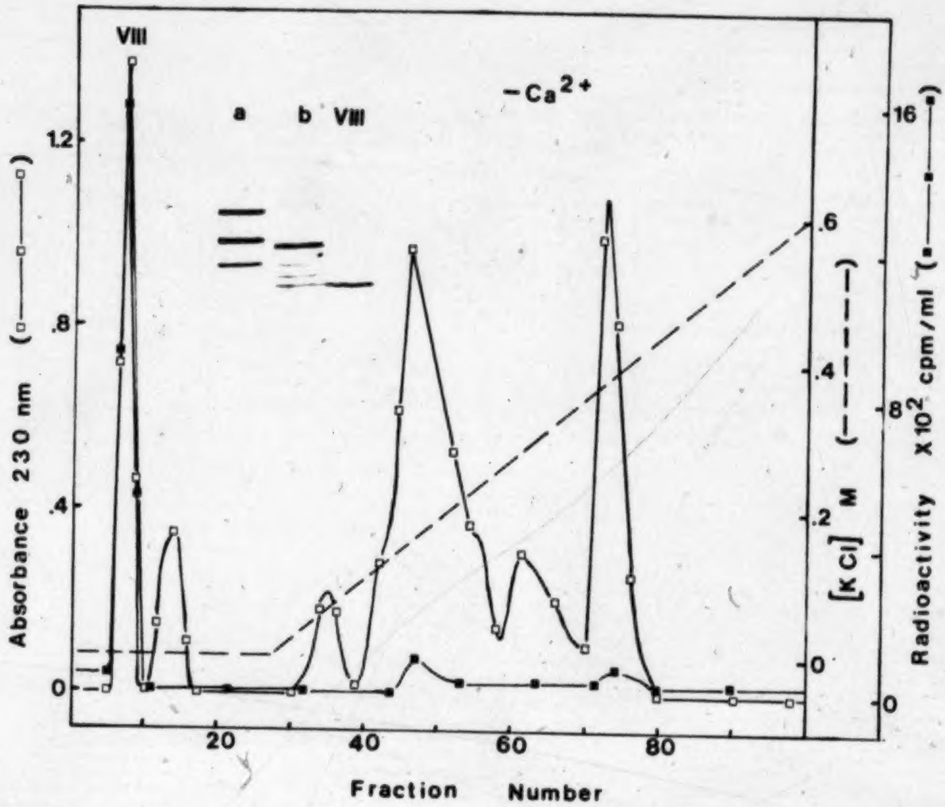

FIG. 6.6. Chromatographic separation of a 15 min limited chymotryptic digest of CM-Tn-AGC (Peak III, Fig. 6.4) on DEAE-Sephadex A-50. $-Ca^{2+}$ refers to the absence of calcium in the buffer during photolysis. Similar results were obtained in the presence of Ca^{2+} The column (1.6 x40 cm) equilibrated with 50 mM Tris-HCl, 8 M urea, 1 mM dithiothreitol buffer, pH 7.8, was eluted with a linear salt gradient (-----) from 0 to 0.6 M KCl in the same buffer (150 ml each). The column effluent was monitored for absorbancy at 230 nm (□-□) and radioactivity (■-■). Inset: SDS-urea Laemmli gel of (a) CM-Tn-AGC; (b) CM-Tn-AGC after a limited chymotryptic digest; and Peak VIII.

6. Purification of T2-AGC from a Limited Chymotryptic Digest of CM-Tn-AGC

Recent evidence has indicated that two regions of TnT may be involved in the binding of the Tn complex to TM. From a chymotryptic digest of TnT, fragments T1 and T2 were isolated and identified as residues 1-158 and 159-259, respectively, both of which were bound to TM immmobilized on a Sepharose affinity column (Ohtsuki,1979; Pearlstone and Smillie,1981). Morris and Lehrer (1981) using the fluorescent probe N-(1-anilinonaphthyl-4)maleimide attached to cysteine 190 of TM have shown that T2 enhances the fluorescence of the label while T1 has no effect. To determine the actual region of TnT that contained the radiolabel in our crosslinking experiments a limited chymotryptic digest of CM-Tn-AGC was performed under the conditions described in the "Experimental Procedures". Usually this limited digestion with chymotrypsin resulted in the cleavage of TnT into the two major fragments corresponding to T1 and T2 without significantly affecting the TnC and TnI components as shown by the SDS-urea Laemmli gel electrophoretic pattern (Fig. 6.6, insert). These results are consistent with those reported by Ohtsuki(1979). Separation of the chymotryptic digest of CM-Tn-AGC by DEAE chromatography is shown in Fig 6.6. The majority of the radioactivity was observed in one peak (Fig. 6.6). Amino acid analysis of Peak VIII (Fig. 6.6) was similar to T2 identified by Pearlstone and Smillie(1981). Peak VIII was further purified on a TM-Sepharose affinity column as described in the "Experimental Procedures". Peak VIII was resolved into two components on the TM-Sepharose affinity column. Peak IX (Fig. 6.7) was eluted in the void volume with a 0.1M KCl buffer while peak X (Fig. 6.7) was eluted with 0.5M LiCl buffer. Both peaks IX and X were radiolabelled and identified as T2-AGC by Laemmli gel electrophoresis (Fig 6.7, insert). Both peaks IX and X have similar amino acid compositions as T2 identified by Pearlstone and Smillie (1981). To determine whether peak IX was eluted in the void volume because of column overloading this fraction was rerun on the affinity column and eluted in the identical position. It is possible that the photoaffinity labelling may have modified a portion of T2 so that it no longer binds to the TM-Sepharose column. The amino acid composition of Peak X is shown in Table 2 and compared to the published composition for T2. The higher values of serine and tyrosine can be explained if the chymotryptic cleavage site was at tyrosine 155 rather than tyrosine 158. This difference in the proteolytic cleavage pattern may be explained by the fact that the limited chymotryptic digests in our experiments were performed on whole Tn rather than TnT as done by Pearlstone and Smillie (1981), who also identified 156-259 as a minor cleavage product. Moreover, the mobilities of T2 (gift from Dr. Smillie) and T2-AGC were slightly different on Laemmli gel electrophoresis (Fig. 6.7, insert). This may be explained by the presence of the AGC

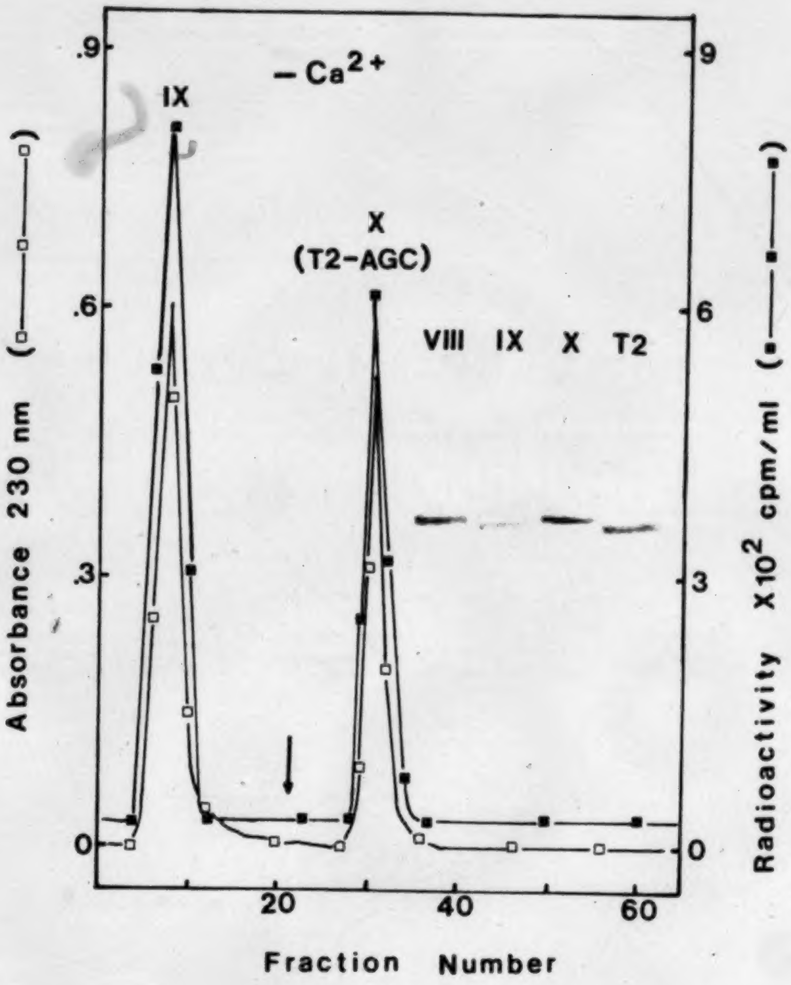

FIG. 6.7. α-TM-Sepharose affinity column profile of Peak VIII (Fig. 6.6) isolated from DEAE-Sephadex A-50 chromatography. -Ca^{2+} refers to the absence of calcium in the buffer during photolysis. Similar results were obtained in the presence of Ca^{2+}. The protein fraction was dissolved in 1 ml of starting buffer and applied to the column (1 x 40 cm) equilibrated with 10 mM imidazole, 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol buffer, pH 7.0. The arrow indicates the start of the 0.5 M LiCl buffer. The column effluent was monitored for absorbancy at 230 nm (□-□) and radioactivity (■-■). Inset: SDS-urea Laemmli gel of Peaks VIII to X and a T2 standard obtained from Dr. Smillie.

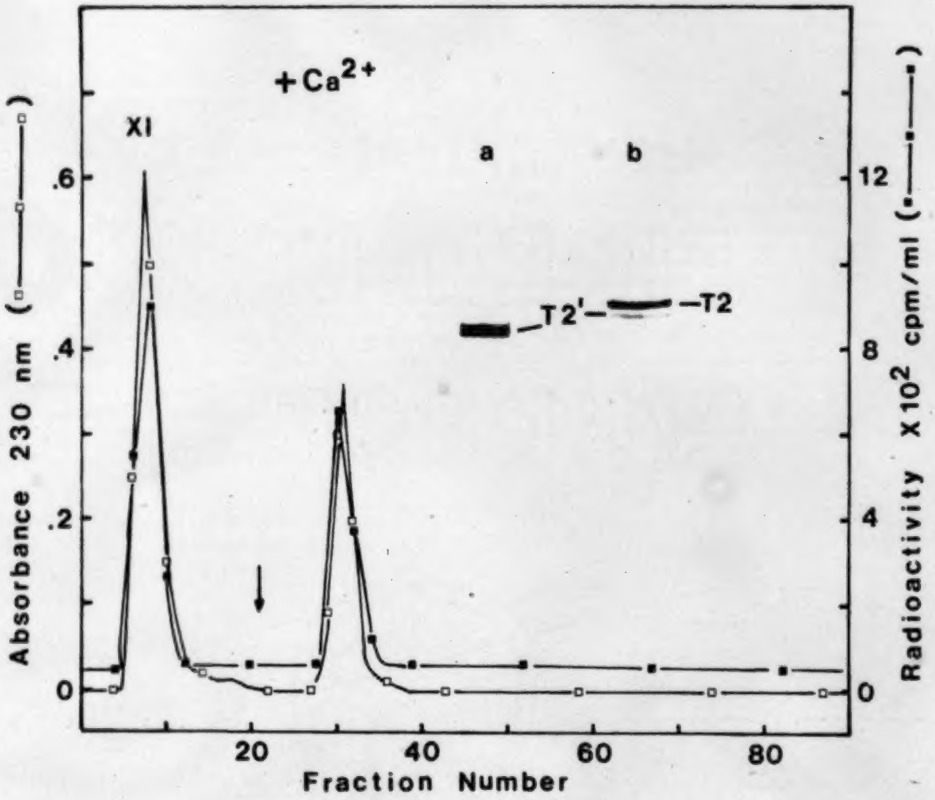

FIG. 6.8. Purification of T2'-AGC on α-TM-Sepharose affinity column. This sample was obtained from a 25 min limited chymotryptic digest of CM-Tn-AGC and isolation of the radioactive peak on DEAE-Sephadex A-50 chromatography. +Ca^{2+} refers to the presence of calcium in the buffer during photolysis. The buffer and column conditions are the same as in Fig. 6.7. The arrow indicates the start of the 0.5 M LiCl buffer. The column effluent was monitored for absorbancy at 230 nm (□-□) and radioactivity (■-■). Inset: SDS-urea Laemmli gel electrophoresis: a, Peak VI; b, Radioactive peak from DEAE-Sephadex chromatography before the affinity column.

moiety. Similar results were obtained when the digest was performed on CM-Tn-AGC isolated from the AGC-TM and CM-Tn complex after photolysis in the presence of Ca^{2+}. The amino acid composition of T2-AGC isolated from this experiment is also shown in Table 6-II.

Recent studies (Ohtsuki,1979; Pearlstone and Smillie,1981) have shown that T2 is very susceptible to further chymotryptic cleavage into two fragments T2' and T3. In one of our experiments (photolysis carried out in the presence of Ca^{2+}) the radioactive peak on DEAE chromatography was shown to contain both T2 and T2' (Fig. 6.8, insert b) which could be separated on TM-Sepharose chromatography (Fig. 6.8). In fact further proteolysis must have taken place during the purification procedure (dialysis and chromatography) since the ratio of T2 and T2' increased in favor of T2' after the TM-Sepharose chromatography in comparison to the intensities on gel electrophoresis after DEAE chromatography (Fig. 6.8). Peak XI (Fig. 6.8) was radiolabelled and showed a single band on SDS- urea Laemmli gel electrophoresis (Fig. 6.8, insert a). The amino acid composition of T2'-AGC is shown in Table 6-II. This result indicates that the region represented by T2' (residues 156-227) of TnT is within 14 Å of cysteine 190 of TM.

TABLE 6-II

Amino Acid Composition of the Purified Radiolabelled Peptides obtained from a Chymotryptic Digest of CM-Tn-AGC[a]

Amino Acid	T2[b]	EGTA	Calcium		T2'[b]
		T2-AGC (Peak X)[c]	T2-AGC (Peak X)[c]	T2'-AGC (Peak XI)[c]	
Asx	9.2(9)	9.3(9)	10.0(9)	6.7(7)	5.6(7)
Thr	4.9(5)	4.2(5)	4.1(5)	2.2(3)	3.2(3)
Ser	2.6(2)	4.2(4)	3.9(4)	2.6(3)	1.7(1)
Glx	13.1(12)	14.9(12)	14.3(12)	12.6(11)	11.3(11)
Pro	0.7(1)	N.D.(1)	N.D.(1)	N.D.(1)	0.9(1)
Gly[d]	5.8(6)	6.1(7)	6.1(7)	3.7(3)	2.4(2)
Ala	9.0(9)	9.1(9)	9.8(9)	7.1(5)	5.5(5)
Val	2.6(3)	3.4(3)	2.2(3)		
Met	2.4(3)	2.8(3)	2.7(3)	2.0(1)	0.9(1)
Ile	2.9(3)	3.2(3)	3.3(3)	2.1(2)	2.0(2)
Leu	9.6(10)	10.4(10)	10.7(10)	7.1(9)	8.3(9)
Tyr	1.9(2)	2.6(3)	3.1(3)	1.3(3)	1.8(2)
Phe	2.9(3)	2.3(3)	2.7(3)	1.6(2)	1.9(2)
His	1.2(1)	1.4(1)	1.6(1)	1.0(1)	1.0(1)
Lys	19.1(21)	17.4(21)	16.6(21)	12.4(15)	13.5(15)
Trp	N.D.(2)	N.D.(2)	N.D.(2)	N.D.(1)	N.D.(1)
Arg	8.9(9)	9.6(9)	9.7(9)	7.4(6)	6.1(6)
Assigned position in sequence	159-259	156-259	156-259	156-227	159-227

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[a] Residues/molecule: Integral values in parentheses were obtained from the sequence of TnT (Pearlstone et al., 1977); N.D., not determined.

[b] Amino acid composition of fragments T2 and T2' published by Pearlstone and Smillie (1981).

[c] Photochemical cross-linking of the CM-Tn and AGC-TM was carried in the presence of either 1 mM EGTA or 3 mM $CaCl_2$.

[d] The radioactive cross-linker AGTC contains one glycine residue per molecule.

D. Conclusions

Using the photoaffinity probe AGTC attached to cysteine 190 of TM the present studies lead to the following conclusions: firstly, when the crosslinking was carried out in either the presence or absence of calcium only TnT was radiolabeled. This indicated that only TnT was in the vicinity of cysteine 190 of TM (within 14 Å). Secondly, the increase of 1.7-fold in crosslinking to Tn in the absence of Ca^{2+} compared to the presence of Ca^{2+} suggests that there is a calcium sensitive conformational change in the binding region of TnT around cysteine 190. A tightening of the complex about cysteine 190 could explain the decrease in reaction of the arylnitrene with solvent in the absence of calcium. Thirdly, a limited 15 min chymotryptic digest of CM-Tn-AGC isolated from the CM-Tn-AGC-TM complex formed in the presence or absence of calcium resulted in the isolation of T2-AGC (residues 156-259). In both cases T2-AGC was shown to bind to a TM-Sepharose affinity column. Fourthly, a more extended proteolysis of CM-Tn-AGC (25 min digest with chymotrypsin) resulted in the isolation of T2'-AGC (residues 156-227). Interestingly, T2' has been shown to bind to TnC in the presence of Ca^{2+} and to TnI (Ohtsuki,1980). When the photoaffinity probe AGTC was attached to cysteines 48 and 64 of TnI, crosslinking to the region 176-230 of TnT was observed (see Chapter VII). Lastly, the use of the SH-directed photoaffinity probe AGTC has been successfully demonstrated.

The synthesis of a ^{14}C-labelled crosslinking reagent is now in progress to aid in the isolation of radioactive peptide fragments from a proteolytic cleavage of T2 and in the sequence analysis of these peptides.

VII. PHOTOCHEMICAL CROSS-LINKING BETWEEN RABBIT SKELETAL TROPONIN Subunits

A. The Importance of SH Groups in the Troponin Complex

Several studies have indicated that the sulfhydryl (SH) groups of TnI are probably involved in the site of interaction between TnI and TnT. Troponin, reconstituted with oxidized TnI, produced very little calcium sensitivity in the actomyosin ATPase and this activity was restored by adding dithiothreitol to the complex(Horwitz et al.,1979). Also, the oxidized TnI will not bind to TnT whereas the reduced TnI will (Horwitz et al.,1979; Hincke et al.,1979) Furthermore, in Chapter III, we found that cysteine 133 of TnI in native rabbit skeletal troponin was accessible to alkylation with iodoacetamide in the presence and absence of Ca^{2+} while cysteine residues 48 and 64 of TnI and cysteine 98 of TnC were inaccessible to modification. These results indicated that cysteine residues 48 and 64 of TnI and cysteine 98 of TnC were either sterically blocked at the sites of interaction between troponin subunits or buried in their own troponin subunit by the conformational changes induced upon the binding of another subunit at the site removed from the vicinity of the sulfhydryl groups. In this study, we describe our approach to selectively modify cysteine residues 48 and 64 of TnI with the heterobifunctional photoaffinity probe AGTC; photolysis of the reconstituted troponin complex; isolation of the covalently crosslinked complex and the identification of the regions of troponin in close proximity to cysteines 48 and 64 of TnI.

B. Experimental Procedures

1. Preparation of AGC-(CM-TnI)

The introduction of the photoaffinity probe (arylazide structure) into S-carboxamidomethylated troponin I (CM-TnI was prepared from CM-Tn as described in Chapter III) was carried out as follows: CM-TnI (30 mg, 1.5 umole) was dissolved into 5.0 ml of 8 M urea, 50 mM Tris-HCl, 0.2 M KCl, 10 mM dithiothreitol, 1 mM EGTA buffer, pH 8.0, and stirred for 2 hours at room temperature. This step ensured complete reduction of the free sulfhydryl groups in troponin I. Dithiothreitol, urea, Tris-HCl and EGTA were removed from the protein by gel filtration on a Sephadex G-25 column (1.5 x 100 cm) eluted with 1 mM HCl, 0.1 M KCl buffer. To the protein fraction (20 ml) was added solid ultra pure urea to give a final concentration of 6 M, and 70 mg of solid AGTC so that the molar ratio of CM-TnI to the photoaffinity probe was 1:100. The reaction mixture was adjusted to pH 8.0 with 2 M Tris solution and stirred in the dark at room temperature for 6 hr, and then the solution was lyophilized. The lyophilized material was dissolved into 8 ml of distilled water and stirred for 2 hr. The excess AGTC was removed by gel filtration on a Sephadex G-25 column (1.5 x 100 cm) previously equilibrated with 1 mM HCl, 0.1 M KCl buffer. The incorporation of the reagent was determined by radioactivity measurements and amino acid analysis as described previously in Chapter IV. Usually, 95-100% modification was achieved.

2. Reconstitution of Troponin

The following method was used to reconstitute the troponin complex from TnC, TnT and AGC-(CM-TnI). Troponin T (45 mg, 1.5 umole) was dissolved into 9 ml of 1 mM HCl, 0.2 M KCl buffer and added into a troponin C solution (27 mg, 1.5 umole in 7 ml of 100 mM Tris-HCl, 0.1 M KCl, 10 mM dithiothreitol, 3 mM $CaCl_2$ buffer, pH 8.0). The protein solution was stirred for 1 hr at room temperature and then dithiothreitol was removed from the protein complex by gel filtration on a Sephadex G-25 column (1.5 x 100 cm) previously equilibrated with 100 mM Tris-HCl, 0.1 M KCl, 3 mM $CaCl_2$ buffer, pH 8.0. To this freshly reduced TnC-TnT complex solution (35 ml) a solution of AGC-(CM-TnI) (1.5 umole/30 ml), which was prepared as described above, was added dropwise over a 3 min period. The reconstituted troponin was stirred under nitrogen at 4°C for 30 min, transferred into the photolysis reaction vessel and saturated with nitrogen for a further 30 min before the photolysis.

3. Isolation of Covalently Cross-linked TnT-AGC-(CM-TnI) Complex

Isolation of covalently crosslinked protein complex after photolysis was carried out on a DEAE Sephadex A-50 column in the absence of reducing agent. The lyophilized material after photolysis (100 mg) was dissolved into 10 ml of distilled water and then dialyzed against 1 litre of

50 mM Tris-HCl, 1 mM EGTA buffer, pH 7.8 at 4°C for 8 hr followed by 500 ml of the column starting buffer (8 M urea, 50 mM Tris-HCl, 1 mM EGTA, pH 7.8) overnight at 4°C. The protein solution was applied to a DEAE A-50 Sephadex column (1.6 x 40 cm), which was equilibrated with the starting buffer. The column was washed with 100 ml of starting buffer at a flow rate of 12 ml/hr and then eluted at the same flow rate with a linear salt gradient consisting of 150 ml each of starting buffer and starting buffer containing 0.6 M KCl. The effluent was monitored for radioactivity and absorbance at 230 nm. The protein peaks were pooled and dialyzed against 1 mM HCl and lyophilized.

4. Isolation of TnT-AGC from Covalently Cross-linked TnT-AGC-(CM-TnI)

The covalently crosslinked TnT-AGC-(CM-TnI) complex isolated from the method described above was dissolved in 4 ml of 8 M urea, 50 mM Tris-HCl, 1 mM EGTA, 10 mM dithiothreitol buffer, pH 7.8, and stirred at 65°C for 15 min followed by 1 hr at room temperature. This step ensured complete reduction of the disulfide bond between CM-TnI and the crosslinker. The protein solution was dialyzed against 500 ml of starting buffer, 50 mM Tris-HCl, 8 M urea, 1 mM EGTA, 1 mM dithiothreitol, pH 7.8 overnight at 4°C, and then applied to a DEAE A-50 Sephadex column (1.6 x 40 cm). The column was eluted as described above except that all buffers contained 1 mM dithiothreitol. The effluent was monitored both by radioactivity measurements and absorbancy at 230 nm. The protein fractions were pooled and dialyzed against 1 mM HCl and 2 mM β-mercaptoethanol and lyophilized.

5. Separation of CB2 and CB3 on DEAE-Cellulose Chromatography

The chromatographic conditions were carried out as previously described by Pearlstone et al.,(1977), except the starting buffer was 50 mM Tris-HCl, 1 mM dithiothreitol, pH 7.8, and the column was 1.6 x 40 cm.

C. Results

1. Selective Modification of TnI with AGTC

In our previous study in Chapter III, we found that cysteine 133 of TnI in native rabbit skeletal troponin was accessible to alkylation with iodoacetamide in the presence and absence of Ca^{2+} while cysteine residues 48 and 64 of TnI and cysteine 98 of TnC were inaccessible to modification. These results suggested that if cysteine 133 was blocked by carboxamidomethylation in native troponin, CM-TnI could be isolated and modified with AGTC at cysteines 48 and 64. Under the conditions described in the "Experimental Procedures", both these cysteines were completely modified by AGTC within 8 hr. We can assume that AGTC modification of CM-TnI does not affect the biological integrity of troponin, because Sutoh and Matsuzaki (1980) found that the troponin reconstituted from TnT, TnC and alkylated TnI had the same calcium sensitivity as native troponin on the actomyosin ATPase assays.

2. Photochemical Cross-linking of the Reconstituted Troponin

In our control experiment, no crosslinked product was observed when the troponin reconstituted from TnT, TnC and unmodified CM-TnI was irradiated (Fig. 7.1d). However, when the troponin reconstituted from TnT, TnC and AGC-(CM-TnI) was irradiated under the conditions described in the "Experimental Procedures", one major crosslinked product was observed by the Laemmli gel electrophoresis as shown in

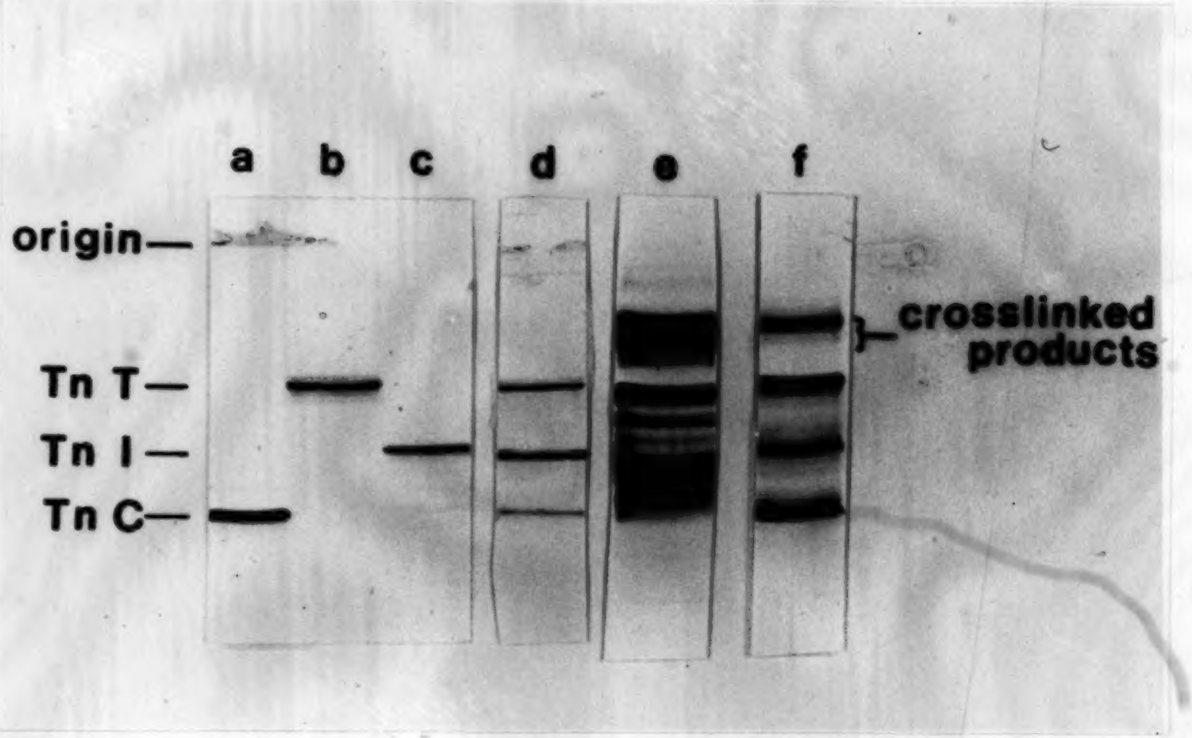


Fig. 7.1. Sodium dodecyl sulfate-urea Laemmli gel electrophoresis of the reconstituted troponin complex formed from TnC, TnT and either CM-TnI or AGC-(CM-TnI). a, TnC + β-mercaptoethanol; b, TnT + β-mercaptoethanol; c, CM-TnI + β-mercaptoethanol; d, the reconstituted troponin complex (TnC + TnT + CM-TnI) after photolysis + β-mercaptoethanol; e, the reconstituted troponin complex (TnC + TnT + AGC-(CM-TnI) after photolysis; f, same as e but β-mercaptoethanol was added.

Figure 7.1e. The apparent molecular weight of this cross-linked product as estimated from the mobility on gel electrophoresis was approximately 54,000, which could correspond to the 52,000 dalton TnI-TnT complex. This result is consistent with the report by Sutoh and Matsuzaki (1980), who also observed the formation of crosslinks between TnI and TnT when the troponin reconstituted from TnT, TnC and methyl 4-azidobenzimidate modified TnI was irradiated in the presence and absence of Ca^{2+}. In the present of β-mercaptoethanol the disulfide bond between the crosslinker and TnI should be reduced and no complex should be observed on gel electrophoresis. However, some irreversible crosslinked product was seen on the gel electrophoresis (Fig. 7.1f). At this time, no definitive explanation can be given for such irreversible crosslinked complex. Moreover, in Figure 1e a weak band was observed with a mobility on gel electrophoresis corresponding to an apparent molecular weight 45,000, which could be the complex of TnI-TnC (41,000) or TnI-TnI dimer (43,000). In the presence of reducing agent, this band was absent. These results are in agreement with the observation reported by Hitchcock, who using dimethylimido esters showed the components of native troponin to be within 6 Å of each other (Hitchcock,1975).

3. Isolation of Covalently Cross-linked Complex

In our previous study we have shown that troponin can be separated into its subunits using DEAE A-50 Sephadex

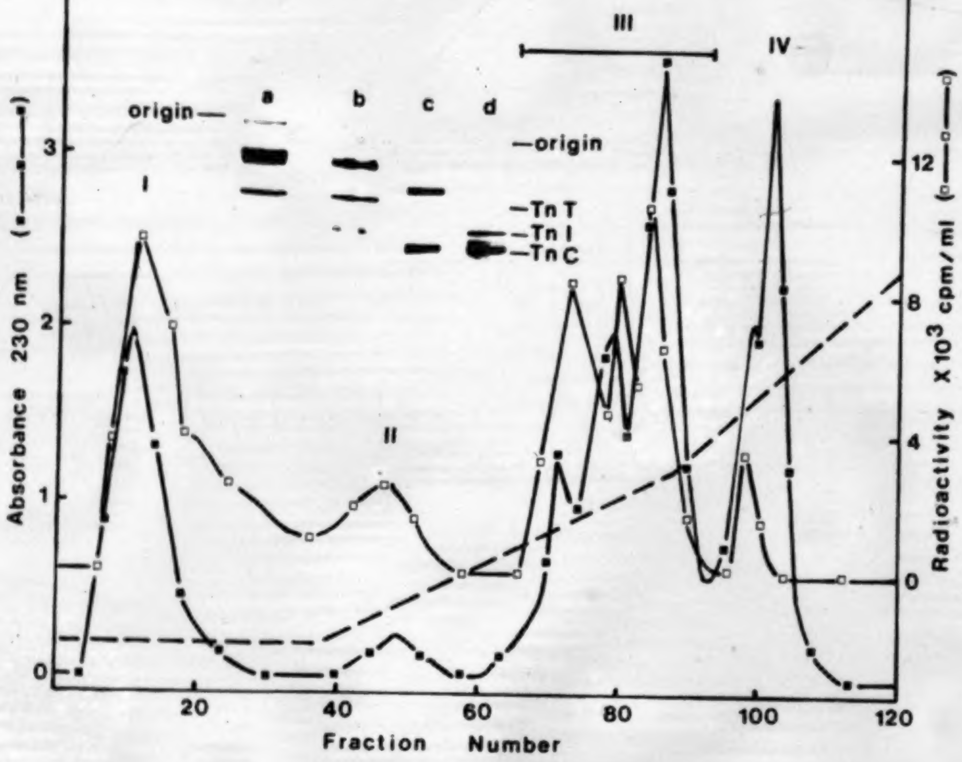


Fig. 7.2. Chromatographic separation of the reconstituted complex of TnC, TnT and AGC-(CM-TnI) after photolysis on a DEAE A-50 Sephadex column (1.6 x 40 cm) in the absence of reducing agent. The chromatographic conditions are described in the "Experimental Procedures". The column effluent was monitored for absorbance at 230 nm (■-■), radioactivity (□-□) and conductivity (---). Inset: SDS-urea Laemmli gel electrophoresis of peak III in the absence and presence of β-mercaptoethanol, a and b respectively; peak IV in the absence and presence of β-mercaptoethanol, c and d respectively.

chromatography in the presence of 8 M urea and 1 mM EGTA. Therefore, the photolyzed troponin complex was separated on DEAE-Sephadex chromatography in the absence of reducing agent (Fig. 7.2) as described in the "Experimental Procedures". The radioactivity measurements in conjunction with SDS-urea polyacrylamide gel electorphoresis indicated that the peaks I and II corresponded to TnI. Since our interest is in the interaction between TnI and other components of troponin, no further studies were carried on peaks I and II. Peak III (fraction 65-93 in Figure 7.2) was a mixture of the covalently crosslinked product, TnT-AGC-(CM-TnI) complex, and the free TnT (Fig. 7. 2,inset a). In the presence of excess β-mercaptoethanol, an additional band corresponding to TnI was observed in the gel (Fig.7.2, inset b). These results suggested that TnT is in close proximity (within 14 Å) to one or both cysteine residues 48 and 64 of TnI. An attempt to separate the TnT-AGC-(CM-TnI) complex from TnT on DEAE A-50 Sephadex with different salt gradients was unsuccessful. Peak IV was a mixture of the covalently crosslinked product TnC-AGC-(CM-TnI) and the free TnC (Fig.7.2, inset c). In the presence of reducing agent, the crosslinked product disappeared and a new band corresponding to TnI was observed (Fig.7.2, inset d). This result indicated that TnC was also within 14 Å of one or both cysteine residues (48 and/or 64) of TnI. In our previous study in Chapter V, we found that TnI was within 14 Å of cysteine 98 of TnC. Sequence analysis of the regions labelled by the probe AGTC will determine whether or not the region of TnC containing cysteine 98 is close to the regions of TnI containing cysteines 48 and 64.

The arylnitrene generated from AGC-(CM-TnI) during photolysis could react either with TnT, TnC, solvent or intramolecularly with CM-TnI to which it is attached(Fig. 5.4). When the arylnitrene reacts with solvent the only attachment of AGC to CM-TnI is via the disulfide bond. In this case the addition of reducing agent would remove the radiolabelled crosslinker. Dialysis against a denaturant (8 M urea) and reducing agent was carried out on each radioactive fraction to remove the crosslinker which had reacted with solvent. Radioactivity measurements before and after the dialysis indicated that 12% and 88% of the crosslinker had reacted with solvent and proteins respectively. This highly productive crosslinking yield (88%) probably reflects the tight binding around the cysteine residues 48 and 64 of TnI in the troponin complex. Furthermore, 55% of the total productive crosslinking was found in peak III, 35% in peak I and II, and 10% in peak IV (Fig.7.2). With the 5.5-fold greater crosslinking yield of the photoaffinity probe to TnT compared to TnC we have concentrated our efforts on identifying the regions of TnT in close proximity to cysteines 48 and 64 of TnI.

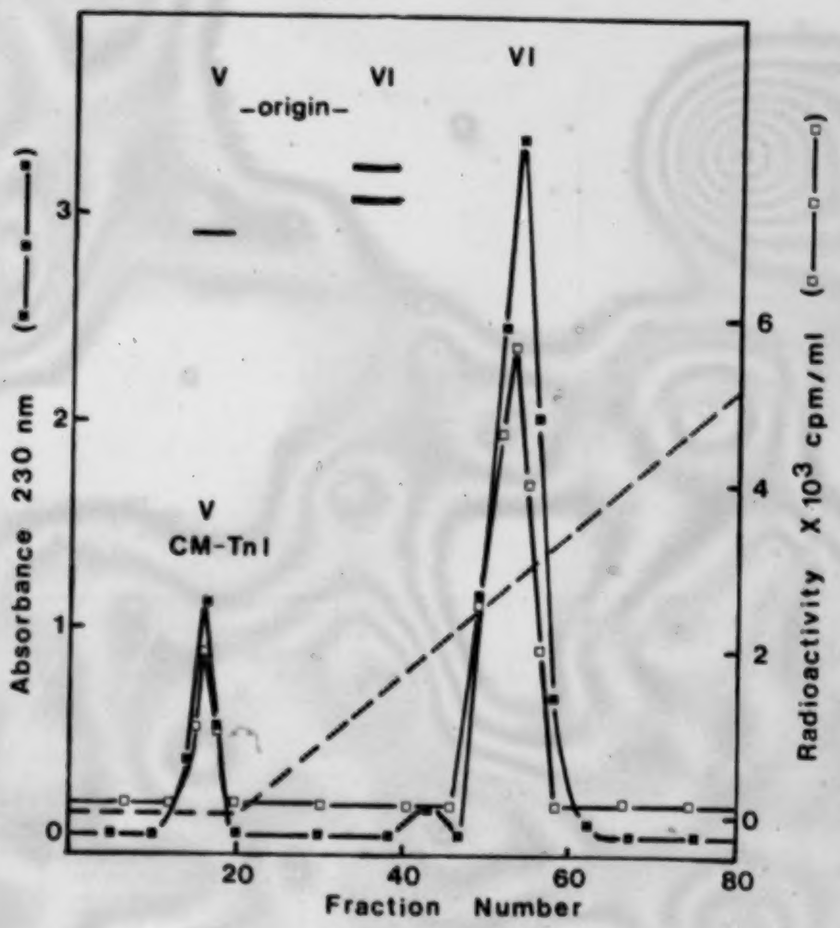


Fig. 7.3. Chromatographic separation of Tn-T-AGC-(CM-TnI) (peak III, Fig. 7.2) on DEAE A-50 Sephadex column (1.6 x 40 cm) in the presence of 1 mM dithiothreitol. The chromatographic conditions are described in the "Experimental Procedures". The column effluent was monitored for absorbance at 230 nm (■-■), radioactivity (□-□) and conductivity (---). Inset: SDS-urea Laemmli gel electrophoresis of peaks V and VI in the presence of β-mercaptoethanol.

4. Isolation of TnT-AGC from the Covalently Linked TnT-AGC-(CM-TnI) Complex

The protein fraction (peak III, Fig.7.2) containing the covalently linked TnT-AGC-(CM-TnI) complex was dissolved in a denaturing medium containing reducing agent to cleave the disulfide bond between TnI and the crosslinker and generate the radiolabelled TnT-AGC and TnI. When the reduced protein solution was rerun on the DEAE A-50 Sephadex column as described in the "Experimental Procedures", two radioactive peaks V and VI were found (Fig. 7.3). SDS-urea polyacrylamide gel electrophoresis showed that only one protein band whose mobility corresponded to TnI was observed in peak V (Fig.7.3, inset). Also, peak V contained 15% of the total radiolabel of peak III (Fig. 7.2). The radioactivity that remained in TnI can be explained by one of the two labels in TnI crosslinking intramolecularly. Peak VI containing 85% of the radiolabel was shown to be TnT-AGC and some irreversible TnT-AGC-(CM-TnI) complex on gel electrophoresis (Fig. 7.3, inset). TnT-AGC was further purified using gel-filtration on P-200 Bio-Gel column (2.6 x 100 cm). Fraction 53-65 contained essentially pure TnT-AGC (Fig. 7.4, inset e). To obtain more TnT-AGC, fractions c and d were combined and rerun on the P-200 Bio-Gel column. This purified TnT-AGC was used to locate the region of TnT within 14 Å distance of one or both cysteine residues of TnI.

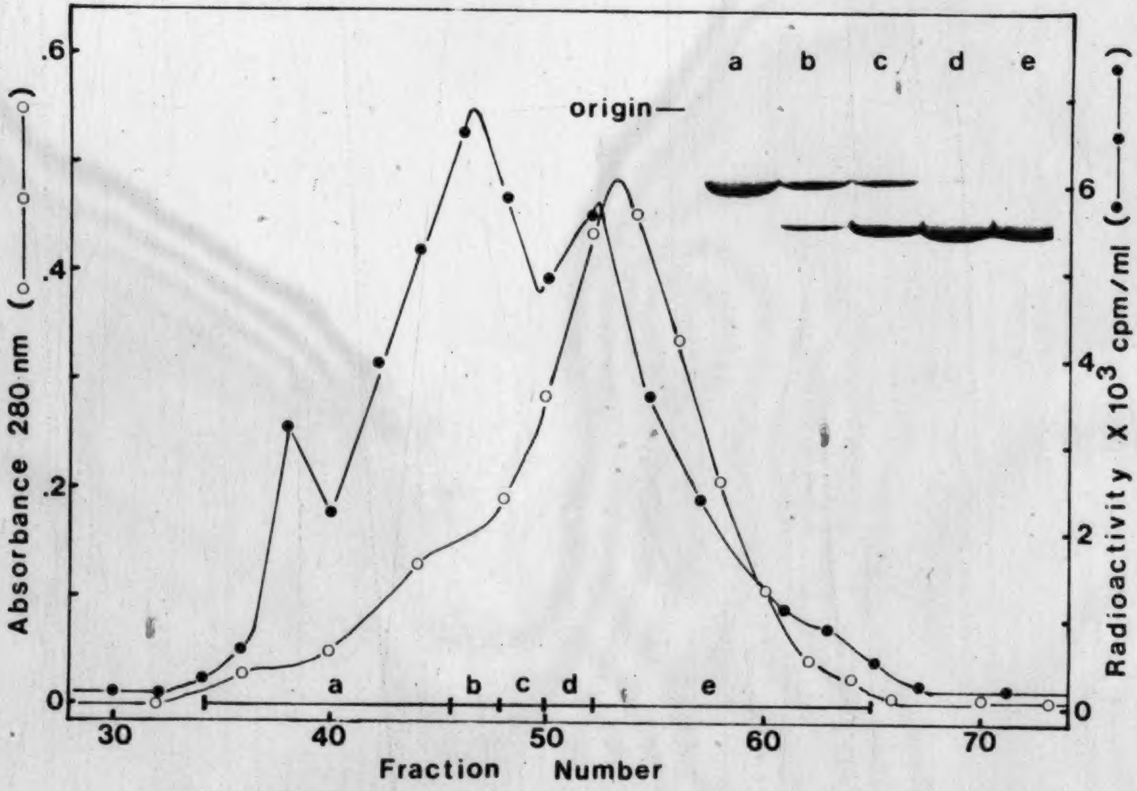


Fig. 7.4. Chromatographic separation of TnT-AGC and TnT-AGC-(CM-TnI) (Fig. 7.3, peak V) on Biogel P-200. The column (2.5 x 100 cm) was equilibrated with 5% formic acid at a flow rate of 10 ml/hr. The column effluent was monitored for absorbance at 280 nm (o-o) and radioactivity (●-●). Inset: SDS-urea gel electrophoresis of fractions a, b, c, d and e in the presence of β-mercaptoethanol.

5. The Region of TnT within 14 Å of cysteines 48 and 64 of TnI

Pearlstone *et al*. (1976) have shown that the cyanogen bromide fragments of TnT can be separated on gel filtration using Sephadex G-75. The cyanogen bromide cleavage of TnT-AGC was carried out as described in the "Experimental Procedures" and the separation of the fragments is shown in Figure 7.5. The radioactivity measurements in conjunction with 12.5% SDS-urea polyacrylamide gel electrophoresis indicated that several peaks contained radiolabel. Fraction 39-45 containing 15% of the total radioactivity was a single band on SDS gel electrophoresis (Fig. 7.5, inset a). This fraction resulted from incomplete CNBr cleavage and because of its size no further characterization was carried out. Fraction 46-58 containing 23% of the total radioactivity consisted of two major peptides on gel electrophoresis corresponding to a mixture of CB2 and CB3 fragments (Fig. 7.5, inset b). Fraction 59-64 containing 33% of the total radioactivity showed a single band on gel electrophoresis (Fig. 7.5, inset c) and the amino acid composition indicated that this peptide was CB4 (residues 176-230) of TnT as identified by Pearlstone *et al*. (1976) (Table 7-I). Fractions 81-92 and 93-104 were small peptides corresponding to CB5, CB6 and CB7 as identified by Pearlstone *et al*. (1976) and contained no radioactivity. Fraction 112-119 was nonpeptide in nature as indicated from the amino acid analysis. One explanation for the appearance of

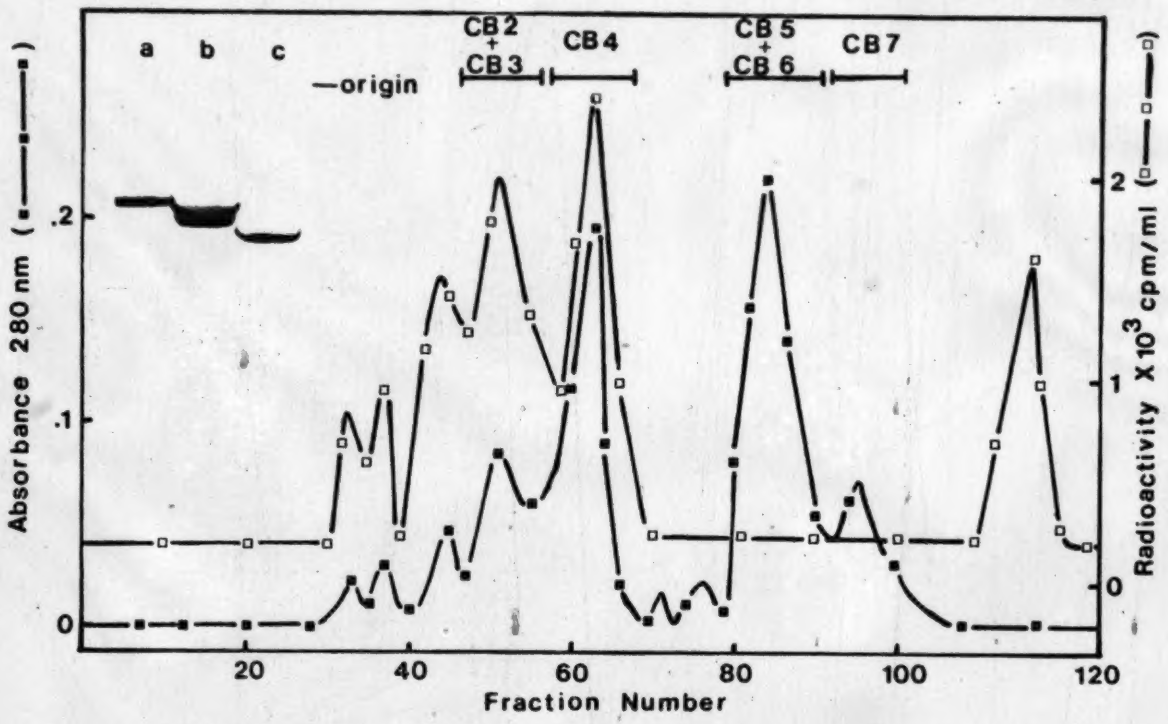


Fig. 7.5. Gel filtration of the CNBr fragments of TnT-AGC. The chromatographic conditions are described in the "Experimental Procedures". The column effluent was monitored for absorbance at 280 nm (■-■) and radioactivity (□-□). Inset: SDS-urea 12.5% gel electrophoresis of fractions 39-45 (a), 46-58 (b) and 59-64 (c).

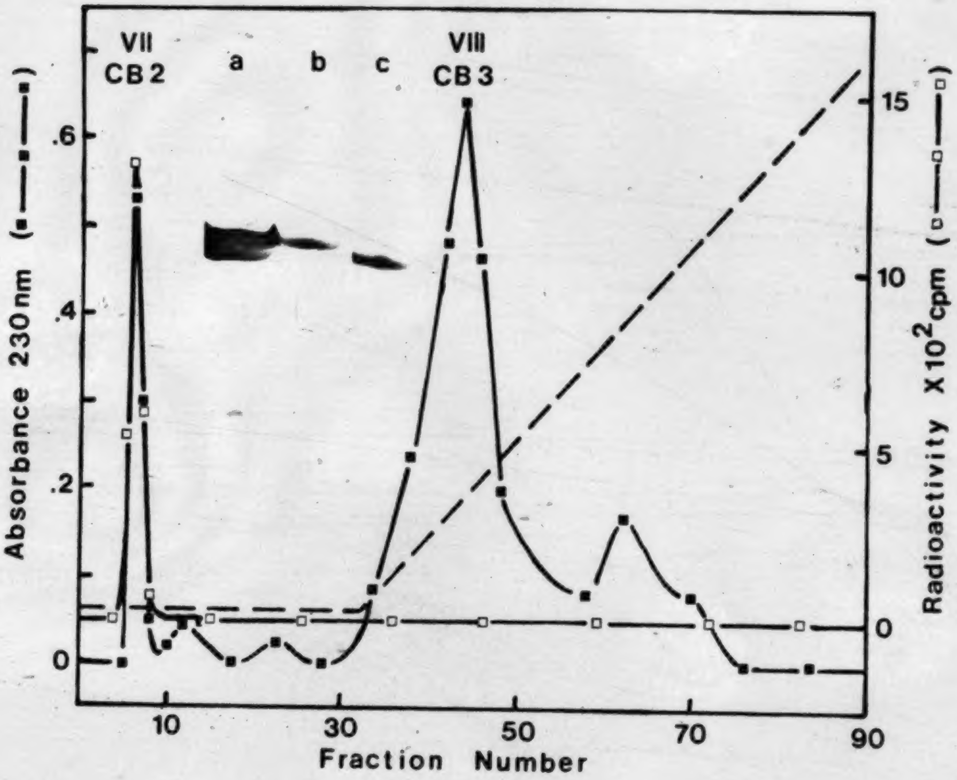


Fig. 7.6. Chromatographic separation of CB2 and CB3 (Fig. 7.5, fraction 46-58) on DEAE-cellulose. The chromatographic conditions are described in the "Experimental Procedures". The collumn effluent was monitored for absorbance at 230 nm (■-■), radioactivity (□-□) and conductivity (---). Inset: SDS-urea 12.5% gel electrophoresis of CB2 + CB3 (a), peak VII (b) and peak VIII (c).

radioactivity at this position is that some of the covalent linkage generated from the nitrene is chemically labile to the cyanogen bromide cleavage conditions. Fisher and Press (1974) also found significant losses of nitrene-derived label during treatment with cyanogen bromide.

CB2 and CB3 were resolved on DEAE-cellulose chromatography as previously described by Pearlstone et al. (1977). As seen in Fig. 7.6, the only radiolabelled peptide was identified as CB2 (residues 71-151) based on SDS-urea gel electrophoresis (Fig. 7.6, inset) and amino acid composition (Table 7-I).

TABLE 7-I
Amino Acid Composition of the Purified Radiolabelled Peptides Obtained from the CNBr Cleavage of TnT-AGC[a]

Amino acid	CB2[b]	CB2-AGC	CB4[b]	CB4-AGC
Asx	4.5 (4)	4.7	7.2 (7)	6.9
Thr	-		2.1 (2)	1.2
Ser	2.8 (3)	3.2	1.1 (1)	0.8
Glx	24.4 (24)	23.9	8.7 (8)	8.8
Pro	-	-	1.0 (1)	N.D.
Gly[c]	-	1.1	1.2 (1)	1.7
Ala	11.7 (11)	11.0	2.4 (2)	2.8
Val	0.9 (1)	N.D.	-	-
Ile	2.8 (3)	3.2	2.9 (3)	2.6
Leu	7.2 (7)	6.8	7.7 (8)	7.3
Tyr	-	-	1.9 (2)	1.9
Phe	1.0 (1)	0.7	2.0 (2)	2.6
His	0.9 (1)	0.9	1.0 (1)	1.3
Lys	10.8 (11)	9.5	10.0 (11)	11.0
Trp	-	N.D.	1.0 (1)	N.D.
Arg	14.2 (14)	13.8	3.9 (4)	4.1
HSer	1.0 (1)	1.1	0.9 (1)	0.8
Assigned position in sequence	71-151	71-151	176-230	176-230

[a] Residues/mole: Integral values in parentheses were obtained from the sequence of TnT (Pearlstone et al.,1977). N.D., not determined.

[b] Amino acid composition of fragment CB2 and CB4 published by Pearlstone *et al*. (1977).

[c] The radioactive crosslinker AGTC contains one glycine residue per mole.

D. Discussion

The reconstituted troponin complex (AGC-labelled CM-TnI, TnT and TnC) was photolyzed and separated on DEAE-Sephadex in the absence of reducing agent. The percentage of radiolabel found in TnI, TnI-TnT and TnI-TnC complexes was 35%, 55% and 10%, respectively. These results have shown that both TnT and TnC are within 14 Å to one or both cysteines 48 and 64 of TnI. In this study we have investigated the regions of TnT containing the radiolabelled photoaffinity probe. Of the total radiolabel found in TnT 33% and 23% was found in the two cyanogen bromide fragments, CB4 (residues 176-230) and CB2 (residues 71-151) (Fig. 7.7).

Pearlstone and Smillie (1980) have shown that the cyanogen fragments CB3 and CB5 bind to TnI while CB4 and CB2 do not (Fig. 7.7). The region containing cysteine 48 and 64 of TnI (Fig. 7.8) is mainly negatively charged at neutral pH (15 glutamic and aspartic acid residues compared to 7 arginine and lysine residues) while the region of TnT containing the C-terminal of CB2, CB5 and the N-terminal of CB4 is mainly positively charged (19 lysine and arginine residues compared to 6 glutamic and aspartic acid residues) (Fig. 7.8). Therefore the most likely interpretation of our results is that one of the interaction sites between TnI and TnT is an ionic interaction involving the region around cysteines 48 and 64 of TnI (residues 28-82) with the CB5 region of TnT (residues 135-185). The second binding site between TnI and TnT would involve the CB3 region of TnT with

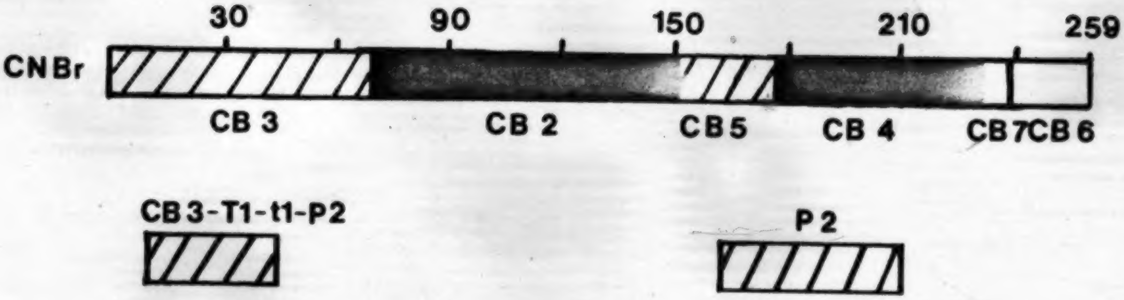

30
90
150
210
259
CNBr
CB 3
CB 2
CB5
CB 4
CB7
CB6
CB3-T1-t1-P2
P2

Fig. 7.7. Position of the fragments in the sequence of TnT. The residue numbers in the complete sequence of TnT are shown in the top bar. The hatch areas represented fragments of TnT found to bind to TnI (19,21). The solid areas represent the cyanogen bromide fragments found to contain the photoaffinity probe transferred from cysteines 48 and/or 64 (present study). The fragment designations are those of Pearlstone and Smillie (1977).

a site on TnI spatially distant from cysteines 48 and 64. Support for this conclusion comes form the fact that CB3 is mainly negatively charged at neutral pH (23 glutamic and aspartic acid residues compared to 9 lysine and arginine residues) and similar in net charge to the region around cysteines 48 and 64 of TnI suggesting that these regions would not interact. In fact CB3-T1-t1-P2 (residues 11-43 of TnT) still binds to TnI and is even more negatively charged at neutral pH than CB3 (12 glutamic acid residues compared to 3 lysine and arginine residues). Our studies would then agree with the fragment studies of Pearlstone and Smillie (1980).

Additional support for the C-terminal region of CB2 and the N-terminal region of CB4 being involved in the TnT-TnI binding site comes from the work of Hitchcock et al.(1981). Their results using a competitive labelling method with acetic anhydride showed that the majority of the lysines with the greatest change in reactivity (comparing the troponin complex with troponin T) are in this region (lysines 143 and 146 in the C-terminal region of CB2; lysines 176, 178 and 185 in the N-terminal region of CB4; and lysines 168 and 169 in CB5 (Fig. 7.8). Similarly, in the native troponin complex lysines 40, 65, 70 and 78 of TnI become less reactive than in TnI itself and lysines 40, 65 and 78 show large reductions in reactivity in the TnI-TnT complex (Hitchcock,1981)

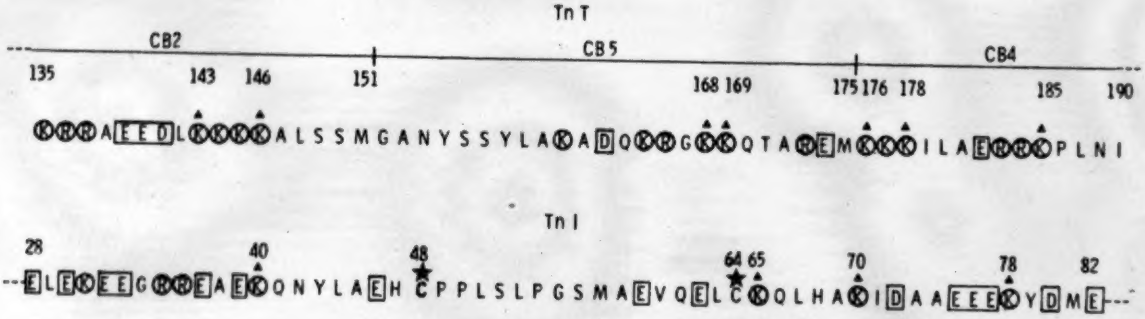

Tn T
CB2
CB5
CB4
135
143
146
151
168 169
175 176
178
185
190
K R R A E E D L K K K K A L S S M G A N Y S S Y L A K A D Q K R G K K Q T A R E M K K K I L A E R R R K P L N I
Tn I
28
40
48
64 65
70
78
82
E L E K E E G R R E A E K Q N Y L A E H C P P L S L P G S M A E V Q E L C K Q L H A K I D A A E E E K Y D M E

Fig. 7.8. Regions of the polypeptide chains of troponin T and troponin I from rabbit fast skeletal muscle involved in interaction. The residue numbers are shown above each sequence. Lysine and arginine residues are circled. Glutamic and aspartic acid residues are boxed. The * symbol denoted cysteines 48 and 64 of TnI that were modified with the photoaffinity probe AGTC. The ▲ symbol represents the lysine residues found by Hitchcock (1981) to show the greatest change in reactivity (comparing the protein itself with the protein in the complex) with a competitive acetic anhydride labelling procedure.

Interestingly, fragment studies have shown that T2' (residues 156-227) of TnT binds to TnC in the presence of Ca^{2+} and to TnI (Ohtsuki,1980). Fragment P2 (residues 159-209) of TnT binds to both TnC and TnI (Pearlstone and Smillie, 1978 and 1980) while CB5 (residues 152-175) binds only to TnI. T2 (residues 156-259) of TnT binds to TM (Ohtsuki,1979; Pearlstone and Smillie,1981) and crosslinking studies have shown that T2' is in the vicinity of cysteine 190 of TM (see Chapter VI). Present crosslinking studies show that cysteines 48 and/or 64 of TnI are in close proximity to CB4 (residues 176-230) of TnT. In other words all components of the calcium regulatory complex (TM-Tn) are interacting with the region 156-227 of TnT. It is now easier to understand how the Ca^{2+}-induced conformational changes in TnC can be transmitted from TnC to all other components of the regulatory complex. With the complexity of interactions in this region it is important to obtain the spatial orientation of the interacting sites. We feel that the heterobifunctional probes will provide us with this information and aid us in our understanding of the molecular events occurring during the contraction process.

VIII. THE QUATERNARY STRUCTURE OF THE REGULATORY COMPLEX

Though there is an enormous amount of evidence accumulating on the sites of interaction between various components of the thin filament regulatory complex (see Chapters I, V, VI and VII), there is little three dimensional structural information available. For example, TnC has been crystallized (Mercola et al., 1975; Strasburg et al., 1980), but no detailed X-ray structural determination has been reported. Thus there is a need for a three dimensional working model of the regulatory complex to aid us in understanding and visualizing how the Ca^{2+}-induced conformational changes in TnC can be transmitted from TnC to all other components of the regulatory complex. For this reason, in this Chapter I would like to discuss how such a working model could be constructed using presently available results.

The individual subunits of the regulatory complex have been constructed under the following constraints:

(i) The secondary structure of each protein is built on the basis of circular dichroism data and the predictive method of Chou and Fasman(1974). The data are summarized in Tables 8-I and 8-II.

(ii) The size and shape of each protein is estimated from hydrodynamic and electron microscopic studies (Table 8-I).

TABLE 8-I
Estimated Helical content (f_α), β-Sheet structure (f_β), Random coil (f_R), and the Size of the Protein in the Regulatory Complex

Proteins	f_α^a	f_β^a	f_R^a	size(Å)[b]
TnC	0.51(0.64)	0.13(0.11)	0.36(0.25)	60-80
TnI	0.29(0.53)	0.20(0.14)	0.51(0.33)	
TnT	0.38(0.37)	0.14(0.10)	0.48(0.53)	100-160
TM	0.96(0.98)	0	0	410

[a] All data are taken from Wu and Yang (1976) except TnT from Pearlstone et al., 1976. The values inside parentheses are based on the predictive method from amino acid sequence (Chou and Fasman, 1974).

[b] The size of proteins is taken from: TnC (Byers and Kay, 1982); TnT (Ohtsuki, 1980; Flicker et al., 1982); TM (Smillie, 1979).

(iii)The folding of each protein is constructed on the basis of the experimental evidence on the sites of enzymatic cleavage and phosphorylation, as well as the relative reactivity of functional groups in each protein.

(iV)The final quaternary structure of the regulatory complex (TM-Tn) is assembled on the basis of the binding studies (see Chapter I, V, VI, and VII).

A. Hypothetical Structure of the Components of the Regulatory Complex

1. Tropomyosin

The structure and physico-chemical properties of tropomyosin have recently been reviewed by Smillie (1979). TM molecules consist of two 284 residue long polypeptide chains arranged in a 410 Å non-staggered coiled-coil structure with 8-9 residues overlap between the NH_2-terminal and COOH-terminal ends of the aggregated molecules when arranged in the thin filaments of the sarcomere.

2. Troponin C

Hydrodynamic studies on bovine cardiac TnC by Byers and Kay (1982) have suggested that TnC could be a flattened molecule of 60-80 Å in diameter. Rabbit skeletal TnC has only one proline residue and a high α-helical content (Tables 8-I and 8-II), and four calcium binding loops. Thus the most probable structure of TnC is shown in Fig. 8.1a.

TABLE 8-II

The Secondary Structure Distribution of Troponin Complex Obtained from Averaging Chou and Fasman Predictive Secondary Structure Parameters

Proteins	The Region Containing		Potential of
	Helices	β-Sheet	Random coil
TnI[a]	5-11	12-24	1-4
	25-43	81-91	44-56
	57-80		105-123
	92-104		132-146
	124-131		153-171
	147-152		
	173-178		
TnC	5-26		1-4
	36-46		27-35
	53-64		47-52
	72-102		65-71
	117-121		103-110
	126-136		137-147
	148-159		
TnT[b]	1-8	107-112	49-67
	26-31	204-211	118-121
	68-102	229-236	152-168
	122-146	238-242	
	164-184		
	216-225		

[a] Adapt from Wilkinson and Grand (1975)

[b] Adapt from Pearlstone et al. (1976)

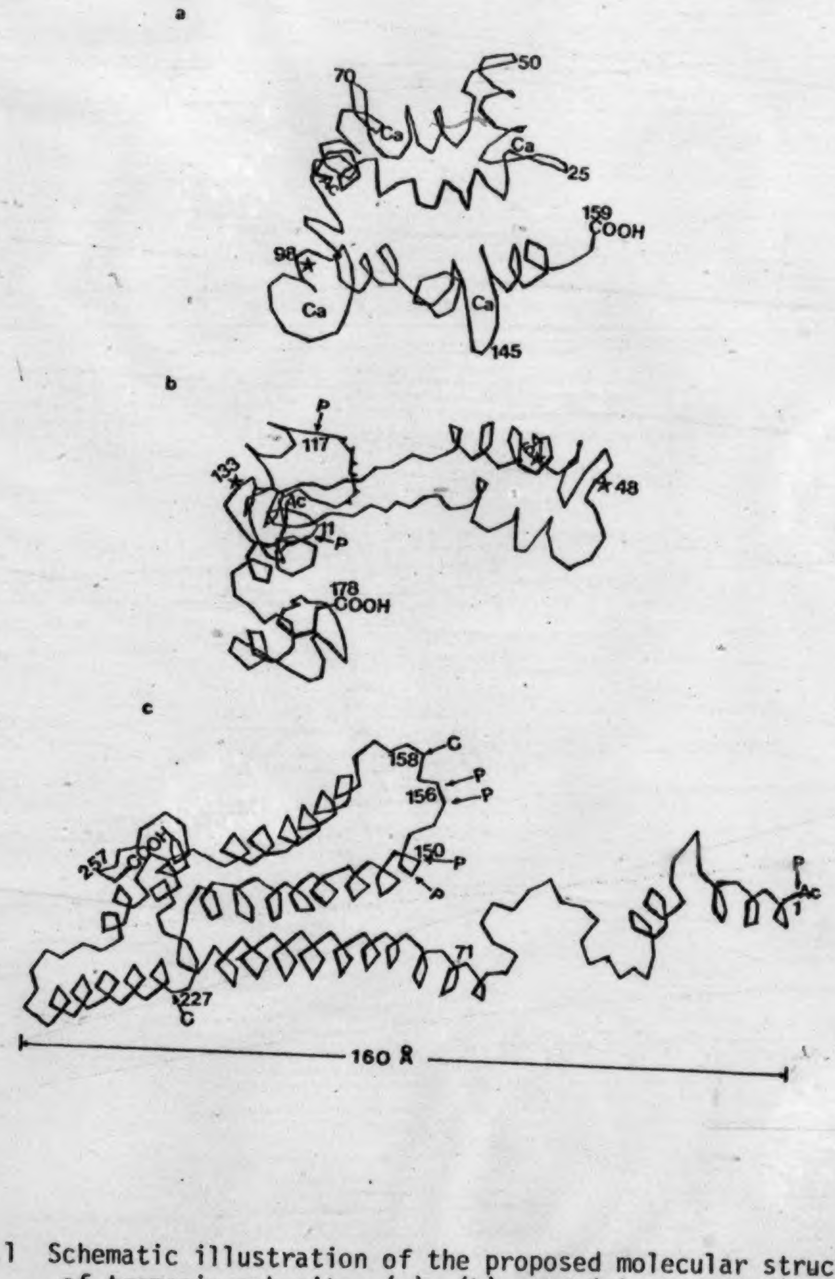

a
70
50
Ca
Ca
25
159
COOH
98
Ca
Ca
145
b
P
117
133
Ac
48
11
P
178
COOH
c
158
C
156
P
P
257
COOH
150
P
P
P
Ac
1
71
227
C
160 Å

Fig. 8.1 Schematic illustration of the proposed molecular structure of troponin subunits. (a), (b), and (c) are TnC, TnI, and TnT respectively. The ★ symbol represents the position of cysteine residues. The ▲ symbol represents the inhibitory region of TnI. P and C are the sites of phosphorylation and chymotryptic cleavage, respectively. Ca indicates the calcium-binding region of TnC. Ac means acetylated N-terminals.

3. Troponin I

Because of poor solubility of TnI, no detailed structural study has been carried out except the primary sequence. According to predicted secondary structure, TnI has several highly helical regions and two long β-sheet regions (Table 8-II). Three proline residues (49, 50 and 54) are found between the two β-sheets, hence the most favorable alignment of the peptide chains would be in an antiparallel fashion (see Fig. 8.1b). The postulated structure of TnI would consist of two structural domains separated by the anti-parallel β-sheets. The molecule would look like a "lobster" in which residues 1-11 and 92-178 compose the head, and residues 25-80 the tail (see Fig. 8.1b). This proposed structure of TnI would have an elongated shape between 80-100 Å in length.

The regions around threonine-11 and serine-117 are exposed so that phosphorylation could be possible. Also the region around residues 96-120 is completely looped out from the main structure so that it could freely interact with actin-tropomyosin molecules. All three cysteine residues (48, 64 and 133) are completely exposed to the surface so that alkylation is possible (see Chapter III).

4. Troponin T

On the basis of immunoelectron microscopic studies (Ohtsuki, 1979 and 1980) and electron micrographs of rotary shadowed TnT-TM molecules (Flicker et al., 1982), TnT has been suggested as a rod-shaped molecule about 100-165 Å in length. Experimental evidence obtained from fragment binding studies between TnT and TM (Mak and Smillie, 1981; Morris and Lehrer, 1981) have revealed that CB1 of TnT (residues 1-151) is bound close to the COOH-terminal end of TM molecule. Photochemical cross-linking results (see Chapter VI) and other studies (Ohtsuki, 1980; Pearlstone and Smillie, 1981; Morris and Lehrer, 1981) have indicated that residues 159-259 of TnT are in close proximity to cysteine-190 of TM. Also this region (residues 152-259) of TnT has been shown to form complexes with TnI and TnC molecules with a globular shape of 100 Å in diameter (Ohtsuki, 1980). In other words, TnT consists of two structural domains: residues 152-259 are thought to bind to the region around cysteine-190 of TM; and residues 1-151 is extended to the COOH-terminal end of TM. Therefore, the most plausible molecular structure of TnT is illustrated in Fig. 8.1c. This proposed structure of TnT is drawn using a modification of the original representation of Pearlstone et al.(1976) to portray the rod-shaped structure of TnT. In this working model TnT is 140 to 160 Å in length depending on the folding at the COOH-terminal end.

The highly helical regions (residues 1-8, 26-31, 68-102 and 210-225) could lie along the same axis and bind to TM molecules in coiled-coil fashion as well as interacting with the head-to-tail overlap region. This binding would link TnT or whole Tn complex to the thin filament. The two helical regions (residues 122-146 and 164-184) are most likely two antiparallel helices in proximity to cysteines-48 and 64 of TnI (see Chapter VII). Also the interaction of these two antiparallel helices with TnI and TnC would explain the changes of reactivity of lysine residues (143, 146, 168, 169, 176, 178 and 185) of TnT found by Hitchcock et al.,(1981). Moreover, the open loop structure around residues 142-167 could provide the sites of specific cleavage by chymotrypsin at tyrosines-155 and 158 (Chapter VI; Ohtsuki,1980; Pearlstone and Smillie,1981), as well as the phosphorylation at serine-149 and 150 and serine-156 and 157 (Moir et al., 1977).

B. Working Model of Regulatory Complex

The working model of the regulatory complex (TM-Tn) is shown in Fig. 8.2. This model is assembled under the followed steps and restrictions:

(i) The two chains of TM are arranged in a coiled-coil structure, and the TnT molecule is constructed as shown in Fig. 8.1c. The NH_2-terminal end of TnT is bound to the COOH-terminal end of TM molecules as discussed above, so that the first and second short helices (residues 1-8 and 26-31) of TnT will interact with the head-to-tail overlap region of TM molecules. Also the random coil region (residues 40-72) of TnT interacts with residues 258-265 of TM such that the effects of CB1 (residues 1-151) of TnT on the iodination of tyrosines-261 and 267 of TM (Mak and Smillie, 1981) could arise from the masking of these residues by the loop structure of residues 33-65 of TnT. The two highly helical regions (residues 68-102 and 210-225) could form a triple stranded coiled-coil with TM at residues 230-265 and 180-195 respectively, leaving cysteine-190 of TM in proximity to the region 159-230 of TnT.

(ii) The "lobster" like TnI molecule is assembled into the TnT-TM complex. The two anti-parallel helices (residues 25-43 and 57-80) of TnI are bound within 14 Å to the two anti-parallel helical regions (residues 122-146 and 164-184) of TnT as described in Chapter VII. From the working model, all negatively charged residues around 30-82 of TnI are well positioned to the positive charge residues around the region

140-185 of TnT. This arrangement fits well to our prediction that one of the interaction sites between TnI and TnT is ionic interaction involving these regions (see the discussion section of Chapter VII). Also, the COOH-terminal end (residues 136-178) of TnI is most likely the second binding site for the TnT molecule, probably the region around residues 200-230 (Pearlstone and smillie, 1980; Hitchcock et al., 1981). The loop structure around residues 96-120 of TnI is not far from TM so that this region of TnI could freely interact with TM-actin molecules. There are two other regions of TnI which are observed in the model to be close to TM, one is the COOH-terminal end (residues 155-178), and the other is the first β-sheet structure (residues 15-27).

(iii) The "flat disc" like TnC molecule is layed on top of TnI-TnT-TM complex. The site III of Ca^{2+}-binding loop (residues 95-115) of TnC is in proximity to the inhibitory region (residues 96-116) of TnI(these two regions are thought to be in close proximity by Perry,1979; and Grand et al., 1982) such that all the charge residues around 84-100 of TnC are well situated for forming ion pairs with the inhibitory region of TnI. This arrangement may explain the prediction and observation by the Perry group (Perry, 1979; Grand et al, 1982) that the interaction between TnC and TnI is electrostatic. The COOH-terminal and the site IV of the Ca^{2+}-binding loop of TnC is located in the vincity of residues 5-25 of TnI, so that threonine-11 of TnI is interacting in a Ca^{2+}-sensitive fashion with TnC (Grand et al., 1982; Katayama and Nazoki, 1982). Furthermore, the region around the sites I, and II of the Ca^{2+}-binding loops of TnC would be expected to interact with the COOH-terminal end of TnI, and the region around residues 200-240 of TnT, which have been shown to bind to a Ca^{2+}-sensitive site of TnC (Pearlstone and Smillie, 1980; Ohtsuki,1980). In order to explain the photochemical cross-linking results obtained for the TnC-AGC-TnI complex (Chapter VII) and the Ca^{2+}-sensitive inhibition effect of TnC on the phosphorylation sites (serines 150 and 156) of TnT (Moir et al.,1977), the NH_2-terminal helix (residues 1-24) of TnC may interact with the helical regions (residues 161-175) of TnT and (residues 57-70) of TnI as seen in the working model (Fig. 8.2).

(iv) The immunoelectron microscopic results of Ohtsuki(1979,1980) allow us to determine the possible polarity of TM-Tn complex on the thin filament with respect to the whole muscle fiber. Ohtsuki(1980) found that antibodies to TnI, TnC and T2 fragment (residues 159-259) of TnT bind farther from the Z-line than antibody to T1 (residues 1-158) of TnT. Hence TM should lie on the actin helix with the COOH-terminal toward the Z-line.

The working model presented in this Chapter is by no means the unique solution of the molecular structure of the regulatory complex. However, from this model a point emerges about the regulatory complex which may be of relevance to its function. That is the sites of interaction between each of the components in the regulatory complex are very likely to involve in the highly helical potential regions. The interactions between these helical regions may explain the well documented fact that there is an approximately one order of magnitude increase in the affinity of TnC for all four Ca^{2+} ions upon interaction with TnI or when calcium binding was measured in native troponin (for review see McCubbin and Kay, 1980).

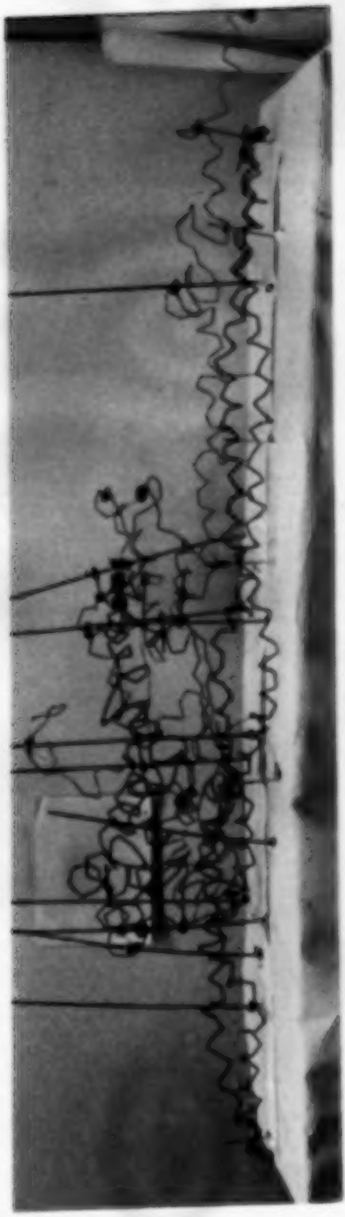
Finally, it is of interest to consider the implication of our model for the regulatory mechanism of skeletal muscle contraction. The asymmetrical shape of the Tn complex as seen in Fig. 8.2 suggests that extensive interaction of TM and TnT may play an important role in the switching process of muscle contraction. For example , helix formation in TnC upon Ca^{2+} binding may be transmitted to both TnI and TnT due to their interactions at the Ca^{2+}-binding regions of TnC as seen in Fig. 8.2., particulary the COOH-terminal half of TnT (residues 159- 240) which interacts extensively with two Ca^{2+}-binding loops of TnC (sites I and II). Hence any changes in TnC molecule upon Ca^{2+} binding will directly induce changes in TnT at this region. Also, the changes that occur in the COOH-terminal end of TnT could be transmitted to its NH_2-terminal end, thus affecting the interaction between the NH_2-terminal end of TnT and the head-to-tail overlap region of TM. This effect could propagate from one TM strand to the next. For this reason the changes at the head-to-tail overlap region of TM may cause a kind of "melting" of TM molecules, thus changing the interaction with each actin molecule like a "bowling effect" and promoting the switching to the "on" state.

Fig. 8.2. A molecular working model of troponin complex bound to the two-stranded α-helical coiled-coil, tropomyosin. Peptide backbone of each molecule is coded in different colors as follows: troponin C (blue); troponin I (red); troponin T (yellow) and tropomyosin (blue). Only the COOH-terminal half of tropomyosin is shown. The ○ , □ , and ▼ symbols represent the positions of cysteine residues, the sites of phosphorylation and the sites of chymotryptic cleavage, respectively.

(a) The side view of the regulatory complex with the COOH-terminal end of tropomyosin on the right-hand side of the picture.

(b) A close-up side view of troponin complex bound in the vicinity of cysteine-190 of tropomyosin.

Note: (b) is from other side than (a); that is TM is running from right to left.



a

b

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